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TOLERANCE OF LARVAE OF DROSOPHILA FOR AMINO ACIDS: TRYPTOPHANE*

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(Received for publication on January 24, 1945)

The failure of a tryptophane-deficient diet to support growth in animals is a well established fact, but relatively few reports have dealt with the growth processes which are modified or controlled by tryptophane. Among these Hammett and Elliot (1935) found that for *Obelia*, tryptophane is a retardant of catabolic activity in that it slows up the process of histolysis associated with senility in that organism. In man (Holt, Albanese, Brunback, Kadji, and Wagerin, 1941) and in dogs (Elman, Davey, and Loo, 1943) absence of tryptophane in the diet results immediately in an increased urinary output of nitrogen. This would be the expected result if catabolism were proceeding at a faster rate because of the lack of tryptophane. As further evidence of retardation of catabolism, Albanese, Holt, Kadji, and Frankston (1943) report a dimunition in haemoglobin and of the protein of blood plasma in rats on a tryptophane-deficient diet. Their data give support to the view that tryptophane is needed for the synthesis of haemoglobin.

Other reported specific effects of tryptophane include a slight lipotropic action on liver fat deposition in rats (Channon, Mills, and Platt, 1943), the protection of rats against pyridoxine deficiency when fed on a low protein diet (Ceredo and Foy, 1944), the inhibition of polysaccharide storage in *Escherichia coli* (Dawson and Happold, 1943), and the enhancement of auxinic activity in tomato tissues (Link and Eggers, 1943). For Obelia, Hammett and Elliot (1935) demonstrated that tryptophane is a specific participant in the processes concerned in differentiation and organization. This latter observation agrees with that of Hoffman and Gudernatsch (1933) on tadpoles

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in which tryptophane favors differentiation. In Obelia, l-tryptophane is also a specific participant, favoring growth initiation. This result is similar to that obtained for certain bacteria (Barker, 1941). Tryptophane is the precursor for the formation of kynurenine, which is involved in pigment formation in the eyes of certain insects (Ephrussi, 1942 review).

Complete lack of tryptophane results in permanent blindness in rats (Curtis, Hauge, Kraybill, 1932). Rats failed to reproduce as well as grow when a tryptophane deficient diet was fed (Albanese, Randall, and Holt, 1943). Drosophilae larvae live for five to seven days without tryptophane but do not grow, rather decrease in size and eventually die (Lafon, 1939).

These few observations serve to point up the need for more knowledge concerning the general and specific rôles of naturally occurring substances in growth. This present paper concerning the rôle of tryptophane in growth processes of Drosophila is one of a series of investigations of the rôle of amino acids in the growth of the fruit fly.

The general material, methods, and procedure of the experiments are described in the first paper of the series (Wilson and Birch, 1944). In these experiments, five grams of fresh Fleischman's yeast was used per 100 cc. of basic culture media. The following concentrations of tryptophane were tested by addition to the basic media, M/25, M/50, M/100, M/250, M/500, M/1000, M/2500, M/5000 and M/10,000. Three experiments using test and control media in each were performed for each concentration.

Discussions and Results

Tables 1 and 2 show the results of addition of concentrations of tryptophane from M/10,000 to M/100 tryptophane to sterile culture media. Length and width of larvae are given in millimeters and percentages of individuals in each instar at each age level are included. No significant difference in length and width between test and control animals occurs at any concentration. There is an indication that moulting between the first and second instars is slightly accelerated by concentrations between M/100 and M/5000, for, in each case a higher percentage of animals have moulted in the test than in the control media. Only two out of the 18 experiments involving these ranges show more test larvae in the first instar at the

TABLE 1

AVERAGE LENGTH AND WIDTH IN MILLIMETERS OF LARVAE TREATED WITH TRYPTOPHANE
AGE IN HOURS FROM MEDIAN HOUR OF OVIPOSITION

Age	Instar	Control No.	Control %	Control Length	Control Width	M/10,000 No.	M/10,000 %	M/10,000 Length	M/10,000 Width	M/5,000 No.	M/5,000 %	M/5,000 Length	M/5,000 Width
50	1	79	60	1.60	.241	26	30	1.55	.234	82	64	1.59	.242
	2	54	40	1.92	.285	62	70	1.97	.270	49	36	2.00	.279
78	2	82	96	3.00	.443	76	80	3.00	.456	96	91	3.01	.460
	3	5	4	3.36	.488	91	20	3.26	.469	10	9	3.28	.467
96	3	120	100	4.50	.627	126	100	4.55	.615	78	100	4.62	.620
120	3	135	100	5.03	.750	80	100	4.91	.770	97	100	5.00	.763
144	3	27	35			58	45			33	34		
	P	50	65			72	55			62	66		
						M/2,500				M/1,000			
50	1	145	69	1.60	.244	32	24	1.63	.250	108	65	1.60	.236
	2	39	21	1.96	.290	99	76	2.03	.285	58	35	2.10	.320
78	2	8	8	3.03	.438	2	1	3.02	.442	44	27	2.94	.417
	3	86	92	3.97	.556	123	99	3.85	.521	118	73	3.88	.548
96	3	100	100	4.68	.611	127	100	4.69	.656	108	100	4.57	.625
120	3	142	98	5.08	.750	75	100	5.00	.752	135	94	4.97	.751
	P	4	2							10	6		
144	3	1	1			1	1						
	P	63	99			62	99			82	100		

P—pupae

TABLE 2

AVERAGE LENGTH AND WIDTH IN MILLIMETERS OF LARVAE TREATED WITH TRYPTOPHANE
AGE IN HOURS FROM MEDIAN HOUR OF OVIPOSITION

		Control				M/250				M/500			
Age	Instar	No.	%	Length	Width	No.	%	Length	Width	No.	%	Length	Width
50	1	90	62	1.55	.238	60	38	1.62	.252	20	11	1.57	.244
	2	56	38	1.93	.277	95	62	1.96	.273	148	89	1.97	.268
78	2	83	46	2.97	.433	82	45	2.98	.423	85	41	2.89	.411
	3	96	54	3.73	.488	98	55	3.68	.511	121	59	3.46	.489
96	3	118	100	4.55	.644	104	100	4.72	.638	73	100	4.32	.618
120	3	77	97	4.88	.726	69	96	4.93	.739	167	100	4.78	.698
	P	3	3			3	4						
144	3	18	17			20	26			16	20		
	P	90	83			55	74			64	80		
						M/100							
50	1	51	30	1.50	.228	20	14	1.48	.219				
	2	119	70	1.96	.270	120	86	1.96	.280				
78	2	159	71	2.97	.430	20	25	2.80	.428				
	3	79	29	3.63	.492	57	75	3.68	.510				
96		102	100	4.90	.691	75	100	4.48	.655				
120		83	100	5.01	.735	87	100	4.84	.701				
144	3	56	36			21	16						
	P	96	64			122	84						

P—pupae

age of 50 hours. The acceleration is not so marked between the second and third instar and does not extend to the period of pupation. No significant difference was noted in the total duration of larval life. Early moulting has been interpreted (Wilson, 1941) as indicating some acceleration of differentiation either through formation of the moulting hormone or by increasing the competence of the reacting tissues. If this interpretation is correct, Drosophila as well as the tadpole and *Obelia* show an acceleration of differentiation in the presence of tryptophane.

That there is no evidence of mass increment in the tests as measured by length and width is of interest for that condition is similar to findings of Hammett and Elliot for *Obelia*. In the present experiments the yeast supplied as food has the equivalent of M/5000 tryptophane combined in its proteins (Meisenheimer, 1921). This amount is sufficient to prevent death and to maintain development. Additional amounts of tryptophane do not increase either length or width of the larvae. Of course, a certain amount of tryptophane is essential before Drosophila will grow at all. In that sense then, it is concerned with increment but here we have a diet that does not supply the optimum protein requirement yet this amino acid unlike arginine (Wilson and Birch, 1944) does not enhance mass increment. In fact, in Table 3, it is shown that a concentration of M/25 tryptophane actually inhibits growth in length (Figure 1) and width. It also retards differentiation, and lengthens the larval period. This concentration is definitely toxic though not completely lethal for 60 per cent of the flies emerged and were able to produce fertile eggs.

This high concentration does upset the kyurenine-pigment-enzyme system, for all the flies treated with M/25 tryptophane have dark brown eyes. Although the difference in size of the larvae is much less pronounced than that of those treated with the higher concentration, 25 per cent of the flies treated with M/50 tryptophane show the same condition for the eye color.

These results may seem to contradict those of Tatum (1939) in which he found that under aseptic conditions tryptophane is without effect in restoring pigment to a vermillion fly, but that in the presence of certain bacteria, brown pigment was produced by addition of tryptophane to the diet. He used 100 mg. of tryptophane per 100 cc. of medium. To give a M/25 concentration, it requires 816 mg. of tryp-

TABLE 3

AVERAGE LENGTH AND WIDTH IN MILLIMETERS OF LARVAE TREATED WITH TRYPTOPHANE
AGE IN HOURS FROM MEDIAN HOUR OF OVIPOSITION

Age	Instar	Control				M/25				M/50			
		No.	%	Length	Width	No.	%	Length	Width	No.	%	Length	Width
50	1	32	73	1.62	.238	24	86	1.41	.212	37	93	1.57	.228
	2	12	27	1.92	.256	4	14	2.08	.300	3	7	2.30	.325
54	1	1	3	1.58	.233	31	100	1.54	.233	10	20	1.53	.229
	2	29	97	1.97	.284					38	80	1.97	.267
77	2	48	67	3.10	.475	46	98	2.66	.387	32	92	2.93	.415
	3	24	33	3.69	.527	1	2	3.75	.525	3	8	3.80	.475
	3	23	80	3.68	.543	72	90	2.74	.404	11	47	2.93	.440
81	2	6	20	2.94	.463	8	10	3.28	.450	14	53	3.49	.515
96	2					4	7	2.66	.384	1	2	2.52	.300
	3	38	100	4.52	.611	52	93	3.53	.495	43	98	4.05	.517
120	3	56	100	5.14	.774	74	100	4.34	.619	27	100	4.71	.758
144	3	4	6			26	97			11	18		
	P	59	94			1	3			48	82		
9 da.	3					3	9						
	P	62	98			24	91			57	97		
	I	1	2							2	3		
10 da.	P	13	20			27				15			
	I	50	80							44			

P—pupae
I—imagoes

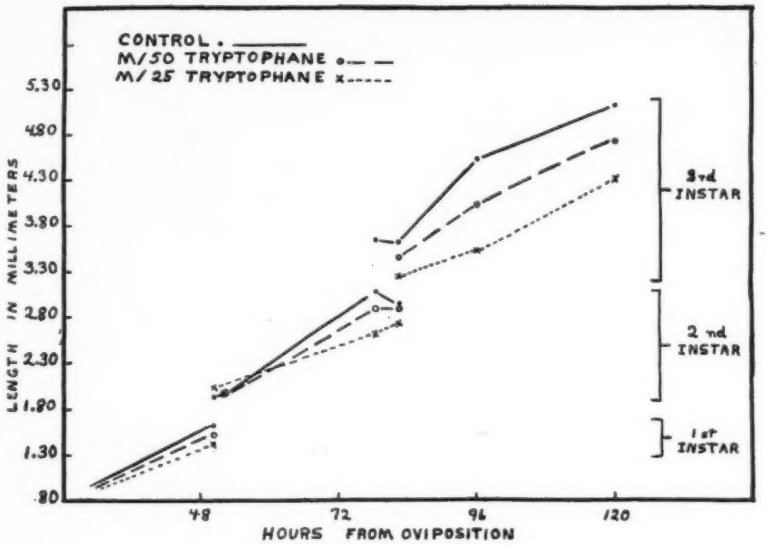

FIGURE 1

RATE OF GROWTH OF LARVAE OF DROSOPHILA TREATED WITH HIGH CONCENTRATIONS OF TRYPTOPHANE

tophane per 100 cc. of culture medium. It seems likely then that even under aseptic conditions, in the presence of sufficient tryptophane Drosophilae can produce a concentration of kyurenine capable of modifying the eye color. Of more than passing interest to the student of growth, looking for fundamental processes modified or controlled by naturally occurring tissue constituents, is the observation of Curtis, Hauge, and Kraybill (1932) that in the rat inadequate protein plus an absence of tryptophane results in permanent blindness. The blindness is characterized by white opaqueness of the eye and loss of the characteristic color and is not related to vitamin *A* deficiency. Tryptophane is concerned then with eye pigmentation in two widely separated phyla, Arthropoda and Mammalia.

Table 4 illustrates another difference between test and control animals. There is a small consistent prolongation of the pupal period in all the animals tested in concentrations from M/5000 to M/100 tryptophane. In every case the percentage of imagoes is less in the

TABLE 4

THE INFLUENCE OF TRYPTOPHANE ON PUPATION (AVERAGES OF 3 EXPERIMENTS)

	9th Day				10th Day			
	Number		Per cent		Number		Per cent	
	P	I	P	I	P	I	P	I
Control	70	22	76	24	10	92	9	91
M/100	97	1	98	2	39	59	40	60
Control	80	28	74	26	16	92	14	86
M/250	67	8	90	10	32	43	42	58
M/500	93	4	96	4	38	59	39	61
Control	18	45	28	72	31	32	49	51
M/1000	63	41	62	38	66	38	64	36
M/2500	77	11	87	13	59	29	65	35
Control	102	15	88	12	71	46	61	39
M/5000	87	4	96	4	65	26	72	28
M/10,000	92	12	89	11	5	99	4	96

P—pupae
I—imagoes

test than in the control on the ninth and tenth day after oviposition.

Since there is no significant difference in the time of pupation in test and control, some process concerned with metamorphosis must be involved in prolongation of the pupal period. According to Needham (1942) the following conditions develop during metamorphosis of a holometabolous insect: loss of weight due to loss of water, utilization of fat and low *R.Q.*, reduction in glycogen, no appreciable change in protein, and histolytic processes accompanied by acidosis. Since there is no difference in size between test and control, it does not seem likely that lack of energy sources, either glycogen or fat, are factors here. In fact it is generally assumed that pupation will not occur until these stores are adequate for completion of metamorphosis.

It seems logical then to suggest that tryptophane here is retarding histolysis just as it did in the case of the *Obelia* hydranth (Hammett and Elliot, 1935). Histolysis of larval tissues is an efficient process and protein building stones are transferred to imaginal structures so that histolysis and imaginal differentiation are simultaneous processes. A similar transfer is made in the case of Obelia where histolysis of senile hydranths is often accompanied by initiation of new growth elsewhere in the colony. Such a parallel is significant in the consideration of the thesis that naturally occurring tissue constituents play some fundamental similar rôle in all protoplasm (Hammett, 1942).

Summary

1. Larvae of Drosophila tolerate tryptophane in concentrations from M/10,000 to M/100 without evidence of toxicity.

2. Acceleration of early larval differentiation as measured by rate of moulting occurs in concentrations of tryptophane from M/5000 to M/100 tryptophane.

3. Prolongation of the pupal period occurs within the same range. It is suggested that the retardation of histolysis of the larval tissues may be responsible for this effect.

4. No effect on mass increment as measured by length and width of larvae is produced by tryptophane at these concentrations.

5. Concentrations of tryptophane, M/50 and M/25, are toxic in that there is a prolongation of larval life and a certain per cent fail to develop.

6. These high concentrations disturb the tryptophane-kyurenine system so that brown eyes are produced in imagoes from wild type Swedish-b parents.

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A STUDY OF INDUCEMENT AND TRANSPLANTABILITY OF SARCOMATA IN RATS*

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In these studies on the inducement and transplantation of tumors rats from 14 different strains, comprising 9,781 individuals, were used. Many of the strains had been under observation in the same colony for 10 to 30 years; none of them for less than five years. Rats of these various strains differ greatly not only in physical characteristics but also in growth rate, behavior, and reaction to stimuli. Whether any of these differences would influence the character and growth of induced tumors, or affect the growth of implanted grafts of induced tumors, was the main problem in mind when these investigations were begun in 1940.

All rats were maintained under like conditions as regards caging, care, temperature, and food. Therefore, none of the results obtained can be attributed to changes in environment or nutrition. The one exception is the experiment on the effect of diet on the time required for the inducement and growth of tumors described in Section *B*, No. 4.

Investigations were carried out in four parts. First, the inducement of tumors in rats of various strains; second, the influence on the development of the primary tumors by various factors such as difference in the strain, age, and sex of the rats treated, the amount and kind of carcinogenic agent employed, and the diet the rats received; third, the transplantation of the induced tumors into rats of their strain of origin and into rats of other strains; fourth, factors influencing the growth of grafts of these tumors transplanted into normal rats of various strains.

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For the inducement of primary tumors each rat was injected with one-half a cubic centimeter of sterile olive oil containing a given amount of a carcinogenic agent. In order to prevent leakage of the injected material the needle was inserted through a sterilized portion of the skin of the cervical region and pushed around into the right axilla. Upon withdrawal of the needle the skin was clamped for a short time. It was difficult to clamp the delicate skin of rats from 5 to 14 days old, therefore some leakage usually occurred when very young rats were injected. By this method of injection inflammation and necrosis were prevented.

Different kinds and amounts of carcinogenic agents were used for tumor inducement: 1:2:5:6—dibenzanthracene, 0.8, 2.0 and 3.3 mgm.; 2:3—benzpyrene, 1 and 4 mgm.; and methylcholanthrene, 1, 3.3, 3.5 and 4 mgm. In all instances tumors that developed were allowed to grow to the size of 50 x 25 x 20 mm., or an equivalent cubic size, before they were recorded and transplanted.

Microscopical preparations were made of each tumor. When examined they all proved to be sarcomata that differed little pathologically. Some were more myxomatous and others more fibrous; a few had an ingrowth of thickened epithelium, possibly malignant, but which was small when compared with the massive growth of spindle cells. The epithelial cells were lost on the first passage of a tumor due to overgrowth of spindle cells.

A. Sarcomata Induced in Rats of Various Strains

1. *King A Inbred Albinos*

The rats used were from the 101st to the 115th generations of a strain known as "King *A* inbred albino" that had been inbred, brother to sister, at The Wistar Institute since 1909 (King, '19). Sarcomata were induced in 90 of these rats. The carcinogenic agents used, the sex and age of the rats when injected, and the time required for development of tumors are given in Table 1. Averages given in this and in following tables were computed from individual data.

Everyone of the treated rats developed a tumor at the site of the injection of the carcinogenic agent.

2. *Gray Norway Rats*

The five litters of gray Norway rats in which 35 sarcomata were

TABLE 1

INDUCEMENT OF SARCOMATA BY HYDROCARBONS IN KING *A* INBRED RATS
(*DBA*, dibenzathracene; *BP*, benzpyrene; *MC*, methylcholanthrene.)

Carcinogenic agent	Amt. inj. in mgm.	Age in days at injection	No. males	Days required for tumor development	No. females	Days required for tumor development
DBA	0.8	10	5	251	2	295
DBA	0.8	14	4	190-224	2	224
DBA	0.8	20	4	197-260	4	248-260
DBA	2.0	11	2	184-225	4	184-280
BP	1.0	63	9	117-137	0	
BP	4.0	114	0		9	121-154
MC	1.0	6	2	155-156	2	146-204
MC	1.0	7	3	128	3	128-130
MC	1.0	8	1	122	2	122-136
MC	1.0	14	3	128	1	102
MC	1.0	21	2	128	7	116-420
MC	3.3	30	10	97-157	9	108-164
			45	Av. 162	45	Av. 177

induced belonged in the 51st to 53rd generation of a strain of wild Norways that had been under domestication since 1919 (King, '39), but had not been inbred, brother to sister, as were rats in the King *A* albino strain.

For inducement of tumors, rats of two litters were injected with dibenzanthracene; those of three other litters with methylcholanthrene. All of the treated rats developed a sarcoma. Data for this series are given in Table 2.

TABLE 2

INDUCEMENT OF SARCOMATA BY HYDROCARBONS IN GRAY NORWAY RATS
(*DBA*, dibenzanthracene; *MC*, methylcholanthrene.)

Carcinogenic agent	Amt. inj. in mgm.	Age in days at injection	No. males	Days required for tumor development	No. females	Days required for tumor development
DBA	2.0	5	6	159-322	3	182-282
DBA	3.3	6	5	147-258	3	180-233
MC	1.0	8	3	126-360	4	149-360
MC	4.0	31	2	135-168	3	129-220
MC	3.5	43	2	117-180	4	177-275
			18	Av. 198	17	Av. 223

3. *Crosses between Gray Norway and Inbred Albino Rats*

Three litters of rats were used for this experiment. One (1) was cast by a gray Norway female that had been mated with an inbred

albino male. The second litter (2) was obtained by mating an F_1 female (gray Norway *x* albino) to an albino male, and the third (3) was the progeny of an F_1 female and a gray Norway male.

The 21 rats in these litters were injected with either dibenzanthracene or methylcholanthrene when from 63 to 84 days of age. Sarcomata developed in all of the injected rats and grew to the standard size within 128 to 389 days (Table 3).

TABLE 3

INDUCEMENT OF SARCOMATA BY HYDROCARBONS IN CROSSES BETWEEN GRAY NORWAY AND INBRED ALBINO RATS

(*MC*, methylcholanthrene; *DBA*, dibenzanthracene.)

Litter number	Carcinogenic agent	Amt. inj. in mgm.	Age at injection (days)	No. males	Days required for tumor development	No. females	Days required for tumor development
1	*MC*	3.5	84	5	128-187	1	258
2	*MC*	3.5	80	7	170-224	1	154
3	*DBA*	3.3	63	4	154-259	3	162-389
				16	Av. 177	5	Av. 239

4. *Rats of Mutant Strains*

Tumors were induced in rats of nine different mutant strains. Four of these strains (ruby-eyed dilute, albino, curly, and cinnamon) originated in the stock of gray Norway rats maintained in King's colony (King, '32; '39). The F_2 generation of a cross between cinnamon and black rats provided the non-agouti brown or chocolate strain (King, '39). Other mutant stocks used originated elsewhere and were sent to The Wistar Institute through the courtesy of those who discovered them or had obtained them for scientific study. Pink-eyed yellows (Castle, '14) were sent by Dr. W. E. Castle who also furnished the wobbly stock discovered by Dr. Amy Daniels (Castle, King, and Daniels, '41); blue rats came from Dr. Elmer Roberts (Roberts, '29); black curly$_2$, from Dr. P. W. Gregory (Gregory and Blum, '36); and the New Haven albinos from Dr. Leon Whitney (King and Whitney, '39).

In making this investigation we hoped to find that the growth rate of sarcomata induced in somatic cells of rats of one mutant strain would differ from that of tumors induced in similar tissue of rats from other mutant strains and in rats of the gray Norway stock.

Conceivably, any difference found might furnish an insight into factors concerned in the origin of sarcomata in general.

In order to induce tumors as nearly alike as possible in rats of the various mutant strains, members of one litter of each strain, and a litter of gray Norways for comparison, were injected when 30 to 31 days old with 4 mgm. of methycholanthrene dissolved in one-half cubic centimeter of sterile olive oil. The time required for the growth of tumors in mutant rats is given in Table 4. This table includes all

TABLE 4

INDUCEMENT OF SARCOMATA BY METHYLCHOLANTHRENE IN RATS OF MUTANT STRAINS

Mutant strains used	No. males	Days required for tumor development	No. females	Days required for tumor development
Ruby-eyed dilute	4	109-165	5	111-173
Albino	2	96-123	4	94-123
Curly	3	115-139	4	121-146
Cinnamon	4	81-177	4	99-158
Chocolate	6	116-151		
Wobbly	3	123-147	3	147-182
New Haven albino	1	115	1	120
Blue	7	108-133		
Pink-eyed yellow	1	108	6	90-118
	31	Av. 126	27	Av. 128

mutant rats used for inducement of tumors. Each rat developed a tumor of the standard size within 81 to 182 days. In members of the litter of the gray Norway rats injected at the same time and with the same kind and amount of carcinogenic agent, tumors grew to the standard size in from 129 to 220 days.

There were no pronounced differences in time required for development of sarcomata in rats of the various mutant strains, although they arose somewhat earlier in pink-eyed yellows, in mutant albinos, and in rats of the blue stock than in rats of other strains used.

B. Influence on Development of Primary Tumors by Various Factors Such as Differences in the Strains of Rats Used, the Amount and Kind of Carcinogenic Agent Employed, and the Age, Sex, and Diet of the Rats

Our records show a marked difference in the time required for development of individual tumors, even in rats of the same litter

treated with the same carcinogenic agent. This difference did not seem to be due to the age of the rats when injected, but was influenced to some extent by other factors.

1. *Strain of Rats*

There was a marked difference in the time of development of tumors induced in rats of the various strains used. In gray Norway rats tumors required from six to seven months to attain the standard size. Growth was slightly more rapid in King *A* inbred albinos and in crosses between gray Norways and albinos. In rats of mutant strains tumors developed in about half the time needed by tumors in the gray Norways (Tables 10 and 11). This difference was particularly marked between tumors induced in litters of mutant strains and those of the litter of gray Norways injected for comparisons when 30 to 31 days old with 4 mgm. methylcholanthrene. Tumors grew to the standard size in rats of mutant strains within 81 to 182 days (Table 4); those in the gray Norways required 129 to 220 days (Table 2).

2. *Carcinogenic Agent*

As is shown in Tables 1 to 4 and in Table 10, rats injected with dibenzanthracene developed tumors somewhat more slowly (147 to 322 days) than did those injected with benzpyrene (117 to 154 days) or with methylcholanthrene (81 to 360 days).

In King *A* inbred albinos sarcomata induced by an injection of dibenzanthracene required a longer time to attain the standard size (50 x 25 x 20 mm.) than did those produced by other agents. In gray Norway rats sarcomata induced by dibenzanthracene also grew more slowly than did those induced by methylcholanthrene.

3. *Influence of Sex*

Regardless of the strain to which the rats belonged, their age when injected or the carcinogenic agent used, the sex of the treated rats had a marked influence on the rapidity of the growth of their primary tumors.

As may be seen from Tables 1 to 4 and Table 12, tumors developed earlier in males than in females in all strains tested. The average time for growth in the 110 males of the 30 litters used, computed

from individual data, was 160 days; the average for the 94 females in these litters was 175 days.

4. *Influence of Diet*

In the course of our investigations we noted that induced sarcomata seemed to develop more slowly in rats that received a diet considered adequate (Purina fox chow) than did tumors that developed in rats which were fed on the standard diet that has been used for many years in King's colony. We decided, therefore, to study the

TABLE 5

THE TIME REQUIRED FOR GROWTH OF A FEW PRIMARY TUMORS AND THEIR GRAFTS

Days required for growth of primary tumors	Days required for growth of first grafts	Days required for growth of later grafts
117	14	10-12
131	22	14-17
132	27	20-27
184	15	12-15
233	41	30-36
239	35	11-14
298	38	15-18

effect of these diets on the 19 members of two litters of inbred albino rats. Ten rats (4 males and 6 females) were fed a balanced diet consisting of corn meal, eggs, meat, fish, fresh vegetables and fruit; nine rats (6 males and 3 females) were given only Purina fox chow. Females were not permitted to breed. All rats were injected with 3.3 mgm. of methylcholanthrene when 30 days old. Results are shown in Table 13.

Sarcomata induced in rats fed on a balanced diet grew to the standard size in from 97 to 164 days, an average of 126.7 days. In the rats receiving Purina fox chow, tumors required from 129 to 159 days for growth to a like size, an average of 136.5 days. Regardless of the diet that the rats received, tumors developed faster in males than in females (Table 13).

C. TRANSPLANTATION OF INDUCED TUMORS

In all susceptible rats grafts of sarcomata grew more or less as they do in tissue cultures, being dependent upon a favorable medium for the multiplication of the cells comprising the graft. On the other

hand, primary tumors induced under the influence of a carcinogenic agent developed from quiescent somatic cells of the host and became transformed into malignant cells that exhibited uncontrolled multiplication. These tumors arose from the host's own tissue and were a part of the host. Transplanted tumors, while retaining the characteristics of the animal from which they originated, were able to continue their uncontrolled multiplication in any favorable medium, even apart from the animal in which they originated as in tissue cultures.

When grafts of various tumors were transplanted into susceptible rats, the time required for their growth to standard size (50 x 25 x 20 mm.) was much shorter than that needed for the inducement and growth of primary tumors to the same size. The time needed for the growth of the graft could be correlated with the number of mitotic figures present in the graft.

After one or two passages in rats of its strain of origin, the graft of each tumor established a more rapid growth rate that remained characteristic of the particular tumor through subsequent passages. Thus there were two classes of tumors, fast growing and slow growing, as is shown by a few examples given in Table 6.

TABLE 6

GROWTH OF GRAFTS FROM TUMORS INDUCED IN KING *A* INBRED ALBINOS IMPLANTED IN RATS OF VARIOUS STRAINS

Strains implanted	Number implants	Number positive growths
Inbred albino	5,000	5,000
Gray Norway	500	111
F_1 Gray Norway x albino	342	342
F_1 backcross to		
(1) Albino	537	537
(2) Gray Norway	361	219
F_2 Gray Norway x albino	209	169
Mutant albino	57	19
New Haven albino	64	22
Pink-eyed yellow	97	47
Curly	22	0
$Curly_2$	10	2
Cinnamon	8	5
Black	51	28
Black piebald	52	16
	7,310	6,517

1. *Transplanting of Sarcomata Induced in King A Inbred Albinos*

Ninety sarcomata were induced in rats of the King *A* inbred albino strain. Grafts of 68 of them were passed through 10 to 50 passages in rats of their strain of origin. For this work more than 5,000 inbred albinos were used. Every one of the grafts grew and not one of the sarcomata regressed.

Grafts of many of these tumors were transplanted into rats of other strains, including black and black piebald rats not used in previous experiments. This black stock originated many years ago in the F_2 generation of crosses between captured wild gray Norway rats and stock albino rats not related to the King *A* inbred albinos (King, '24). Records of this experiment are given in Table 6.

Results of transplantation of individual King *A* sarcomata showed that some of them were strain specific and failed to grow in any rat lacking albino factors. Others were less strain specific and grew in some rats from the various strains tested. Grafts of one sarcomata grew in all the gray Norways, in rats of the various crosses between gray Norways and inbred albinos, and also in a large proportion of the pink-eyed yellows and cinnamons into which they were implanted. Behavior of individual tumors indicated a difference in the tumor and not in the susceptibility of the ingrafted host. When grafts of two tumors that originated in different inbred albino rats were transplanted into a rat of another strain, in some instances the graft of one tumor grew while that of the other did not grow, thus showing a difference in growth energy of the two tumors.

Grafts of tumors that originated in the King *A* inbred albinos grew not only in all of the inbred albino rats, but also in all of the F_1 gray Norway x inbred albinos and in the F_1 backcross to inbred albinos. These hybrid rats apparently contained sufficient albino factors to form a favorable environment for growth of these tumors. Grafts grew in only about 57 per cent of the ingrafted rats of other strains.

2. *Transplantation of the Tumors Induced in Gray Norway Rats*

Grafts of 29 tumors induced in gray Norway rats were transplanted into rats of their strain of origin and also into rats of various other stains. Growth of these grafts is recorded in Table 7.

Tumors induced in gray Norway rats were not 100 per cent

TABLE 7

GROWTH OF GRAFTS FROM TUMORS INDUCED IN GRAY NORWAY RATS IMPLANTED INTO RATS OF VARIOUS STRAINS

Strains implanted	Number implants	Number positive growths
Gray Norway	629	345
F_1Gray Norway x albino	121	51
F_1 backcross to		
(1) Gray Norway	175	79
(2) Albino	253	94
F_2 Gray Norway x albino	9	2
Inbred albino	106	22
New Haven albino	10	6
Mutant albino	11	5
Pink-eyed yellow	12	4
Cinnamon	2	0
$Curly_2$	1	1
Black	4	0
Black piebald	3	0
	1,336	609

transplantable into rats of their own or of any other strain used. This was to be expected since, as previously stated, these rats were not inbred. Rats in none of the other strains used furnished a satisfactory environment for growth of grafts of the majority of gray Norway tumors.

3. *Transplantation of Tumors Induced in Hybrids between Gray Norway and Inbred Albino Rats*

Grafts of 17 of the tumors induced in rats of various crosses between gray Norway and inbred albino rats were transplanted into rats of their strain of origin and also into rats of other hybrid strains. Records for this experiment are summarized in Table 8.

Data in Table 8 show that tumors induced in rats of hybrid strains grew in only a small number of the rats into which they were transplanted. Grafts from tumors inducted in F_1 backcross to Norways proved to be the most transplantable (45.55 per cent positive growth). Grafts grew only in 300 (39.27 per cent) of the other 764 ingrafted hosts.

These findings are in striking contrast to those obtained when tumors induced in King *A* rats were transplanted into rats of various strains (Table 6). They accord with the fact, known for many years, that transplanted tumor cells fail to grow in 100 per cent of

TABLE 8

GROWTH OF GRAFTS FROM TUMORS INDUCED IN HYBRIDS BETWEEN GRAY NORWAY AND INBRED ALBINO RATS IMPLANTED IN RATS OF VARIOUS STRAINS

Strain implanted	Grafts from F_1 Gray Norway x albino		Grafts from F_1 backcross to gray Norway		Grafts from F_1 backcross to albino	
	Number implants	Number positive growth	Number implants	Number positive growth	Number implants	Number positive growth
Gray Norway	66	6	74	41	95	28
Inbred albino	37	2	15	1	42	11
F_1 Gray Norway x albino	33	13	17	5	25	17
F_1 backcross to Gray Norway	54	25	21	7	54	24
F_1 backcross to albino						
Gray	4	0	8	2	20	10
Albino	25	13	27	15	50	26
Gray piebald	8	5	10	5	11	5
Black	10	5	10	7	14	8
Black piebald	9	3	9	4	16	12
	246	72	191	87	327	141

nearly related animals unless the individual in which the tumor arose and that into which it was transplanted were both from a strain that had been inbred for many generations.

4. *Transplantation of Sarcomata Induced in Rats of Mutant Strains*

For this experiment we used rats from nine mutant strains. The sarcomata induced in these rats were transplanted into rats of their own and of other strains. The results obtained are summarized in Table 9.

Sarcomata that arose in litter mates from various mutant strains that had been more or less inbred exhibited marked divergences in growth energy when transplanted into rats of their own and of other strains. Moreover, they failed to show a greater similarity of behavior than did tumors that originated in rats of entirely different strains. In general, these tumors were more transplantable into rats of their strain of origin than into rats of other strains, but they showed no marked affinity for gray Norway rats or for rats of any other mutant strain. However, tumors induced in rats of the New Haven albino strain grew in a greater proportion of rats into which they were transplanted than did those of any other mutant strain. Their grafts grew in 50 (60.24 per cent) of the 83 rats implanted. It is interesting to note that no grafts grew in curly rats. A similar

TABLE 9

GROWTH OF GRAFTS FROM TUMORS INDUCED IN MUTANT RATS IMPLANTED INTO RATS OF THEIR OWN AND OF OTHER STRAINS

Strains implanted	Number implants	Number positive growths
Gray Norway	88	8
Inbred albino	128	38
F_1 Gray Norway x albino Backcross to Gray Norway	63	17
F_1 Gray Norway x albino Backcross to albino	70	5
F_2 Gray Norway x albino	58	4
Mutant albino	62	30
Curly	7	0
Cinnamon	28	20
Chocolate	8	1
Ruby-eyed dilute	4	1
$Curly_2$	8	2
Wobbly	2	1
Pink-eyed yellow	88	42
Blue	17	5
Black	3	0
Black piebald	33	6
	667	180

result was obtained when grafts from tumors induced in inbred albino rats were implanted into curly rats (Table 6).

D. FACTORS INFLUENCING THE GROWTH OF TRANSPLANTED TUMOR GRAFTS

1. *Influence of Age*

When grafts of tumors induced in inbred albino rats were transplanted into rats of their own strain, or into rats of susceptible strains, their growth was not greatly influenced by the age of the host although grafts grew faster in young than in old rats. When implanted into rats of alien strains, grafts from any tumor grew in more of the young rats (20 to 30 days of age) than in older ones (180 to 240 days). Young rats seemed less resistant to the growth of tumors than did old ones.

2. *Influence of Coat Color*

Since all rats injected with a carcinogenic agent developed a tumor at the injection site (Tables 1-4 and 10) it is evident that the development of primary tumors is not influenced by the strain to which the rats belonged or to their coat color. As shown previously

TABLE 10

TIME REQUIRED FOR DEVELOPMENT OF SARCOMATA IN RATS OF VARIOUS STRAINS INJECTED WITH DIFFERENT AMOUNTS OF CARCINOGENIC AGENTS

(*DBA*, dibenzathracene; *MC*, methycholanthrene; *BP*, benzpyrene.)

Carcinogenic agent	Amt. inj. in mgm.	Strain of rats	Age in days at injection	Days required for tumor development
DBA	0.8	Inbred albino	10-20	190-295
DBA	2.0	Inbred albino	11	184-280
DBA	2.0	Gray Norway	5	159-322
DBA	3.3	Gray Norway	6	147-258
DBA	3.3	F_1 (Gray Norway x albino back-cross to gray Norway	63	154-389
MC	1.0	Inbred albino	6-21	122-156
MC	1.0	Gray Norway	8	126-360
MC	3.3	Inbred albino	30	97-159
MC	3.3	Gray Norway	43	117-275
MC	3.5	F_1 Gray Norway x albino	53-84	128-258
MC	3.5	F_1 backcross to albino	80	170-224
MC	4.0	Rats of mutant strains	30	81-182
MC	4.0	Gray Norway	31	135-220
BP	1.0	Inbred albino	63	117-137
BP	4.0	Inbred albino	114	121-154

TABLE 11

TIME OF SARCOMA DEVELOPMENT IN RATS OF DIFFERENT STRAINS

Strains used	Days required for tumor development
Gray Norway	117-360
Inbred albino	97-295
F_1 Gray Norway x Albino	128-258
F_1 backcross to gray Norway	154-389
F_1 backcross to albino	170-224
Mutant	81-182

(Table 6), grafts of sarcomata induced in King *A* inbred albinos grew in all rats of their strain of origin and also in various crosses between gray Norways and albinos, regardless of their coat color. On the other hand, grafts from these tumors grew in only 49 per cent of the 188 albinos of other strains into which they were transplanted. These results seem to indicate that albinism is not a factor that controls the transplantability of tumors that originated in albino rats.

In another experiment sarcomata were induced in rats of three color varieties: gray, albino, and black piebald, obtained by backcrossing F_1 gray Norway *x* albino rats to albinos. Grafts of these

TABLE 12

EFFECT OF SEX ON GROWTH OF INDUCED TUMORS

Strains injected with carcinogenic agent	No. males	Average No. days needed for tumor development	No. females	Average No. days needed for tumor development
Gray Norway	18	198	17	223
Inbred albino	45	162	45	177
Gray Norway x albino crosses	16	117	5	239
Mutant	31	107	27	129
	110		94	

TABLE 13

EFFECT OF DIET ON TUMOR GROWTH

Balanced diet		Purina fox chow	
Days required for tumor growth		Days required for tumor growth	
Males	Females	Males	Females
97	108	129	129
108	129	132	138
108	129	132	159
157	129	132	
	138	138	
	164	138	
Av. 117.5	Av. 132.9	Av. 133.5	Av. 142.0

tumors were then transplanted into rats from litters of various crosses between gray Norway and inbred albinos. Data obtained are given in Table 14.

Grafts of sarcomata induced in rats of the F_1 backcross to inbred albino grew in a considerable proportion of all rats into which they were transplanted regardless of their coat color. Grafts of tumors that iriginated in the albino members of this cross grew in 50.7 per cent of the rats in which they were implanted; grafts of tumors from gray rats grew in 53.3 per cent; and those from black piebalds in 30.8 per cent of their hosts. Each of these tumors grew in a larger proportion of the color variety in which it originated than in rats of other colors from the same litter.

Additional data regarding the effect of coat color on tumor growth were obtained as follows: Grafts from tumors induced in rats of various strains were transplanted into color varieties of rats from

TABLE 14

TRANSPLANTATION OF SARCOMATA INDUCED IN COLOR VARIETIES OF HYBRID BETWEEN GRAY NORWAY AND ALBINO RATS

Strain implanted	Albino		Gray		Black piebald	
	Number implants	Number positive growth	Number implants	Number positive growth	Number implants	Number positive growth
Inbred albino	32	11	2	0	8	0
Gray Norway	63	21	12	11	20	6
F_1 Gray Norway x albino	17	10			8	7
F_1 backcross to						
(1) Gray Norway	36	18	4	0	14	6
(2) Albino						
Color varieties						
Albino	29	23	6	0	15	3
Gray	17	9	1	1	2	0
Gray piebald	6	3	1	1	4	1
Black	9	8			5	0
Black piebald	10	8	4	3	2	1
	219	111	30	16	78	24

F_1 and F_2 crosses between gray Norway and inbred albinos. The results obtained are summarized in Table 15.

As was to be expected from the findings in previous experiments, grafts of tumors induced in inbred albinos grew in a greater proportion (80.4 per cent) of all rats into which they were implanted than did tumors from other strains, and none of them showed an affinity for rats of any particular color. Grafts of tumors induced in gray Norways and in crosses between gray Norways and albinos grew in many albinos and in rats with colored coats. On the other hand, grafts of tumors from mutant rats grew in only a few (6.7 per cent) of the rats implanted regardless of their color. The results obtained in this and in the previous experiment (Table 14) indicate that the coat color of rats is not a factor influencing the growth of tumor grafts.

3. *Influence of Strain*

All of the various mutant strains of rats available for our investigations were derived directly or indirectly from the common gray rat, *Rattus norvegicus*. Diversity in various characteristics of these mutants, aside from coat color, may indicate innate differences in the strains which possibly might affect the growth ability of grafts of tumors transplanted into them.

In the first experiment made to test this assumption sarcomata

TABLE 15

TRANSPLANTATION OF SARCOMA GRAFTS INTO COLOR VARIETIES OF RATS

Strains implanted	Strains supplying tumor grafts: Gray Norway		Inbred albino		Gray Norway x Albino		Mutant	
F_1 Gray Norway x albino backcrossed to albino	No. implants	No. positive growth	No. implants	No. positive growth	No. implants	No. positive growth	No. implants	No. positive growth
Gray	61	30	342	342	32	12	9	1
Albino	125	42	of all	of all	102	54	22	0
Gray piebald	25	11	coat	coat	29	15	14	2
Black	12	6	colors	colors	46	26	16	0
Black piebald	41	16			34	19	9	1
F_2 Gray Norway x albino								
Gray	3	1	90	74			23	0
Albino	3	1	67	51			11	2
Gray piebald	0	0	25	21			5	1
Black	2	0	26	22			10	1
Black piebald	0	0	1	0			1	0
	272	107	551	510	243	126	120	8

were induced by carcinogenic agents in rats of various mutant strains and their grafts implanted in gray Norway rats (Table 16).

TABLE 16

GROWTH OF TUMORS INDUCED IN RATS OF MUTANT STRAINS IMPLANTED IN GRAY NORWAY RATS

Sarcomata from mutant strains	No. gray Norway rats implanted	No. positive growth
Curly	6	0
Wobbly	5	0
Blue	15	0
New Haven albino	4	0
Pink-eyed yellow	13	1
Mutant albino	14	1
Cinnamon	14	2
Chocolate	11	1
Ruby-eyed dilute	8	3
	90	8

None of the tumors induced in rats of the first four mutant strains listed in Table 16 grew in any of the gray Norways into which they were implanted, and those from other mutant strains grew in only eight of the 60 rats tested. Obviously, gray Norway rats do not afford a favorable environment for the growth of tumors induced in mutant rats.

In our second experiment grafts of tumors induced in rats of various strains were implanted in rats of mutant strains (Table 17).

TABLE 17

GROWTH OF GRAFTS OF TUMORS INDUCED IN RATS OF VARIOUS STRAINS TRANSPLANTED INTO RATS OF MUTANT STRAINS

Mutant strains implanted	No implants	No positive growth
Curly	29	0
Mutant albino	130	54
New Haven albino	80	34
Pink-eyed yellow	197	93
$Curly_2$	19	5
Black	58	28
Black piebald	88	22
Blue	17	5
Cinnamon	38	25
Chocolate	8	1
Ruby-eyed dilute	4	1
	668	268

The results of this experiment differ greatly from those summarized in Table 16, since tumors grew in 40.1 per cent of the mutant rats into which grafts were implanted.

The most striking result obtained was that rats of the curly strain proved to be resistant to the growth of grafts of all tumors implanted into them, even to those that originated in rats of their own strain. In all of the experiments described in this report, tumors induced in curly rats have failed to grow in rats of alien strains. This finding indicates that curly rats are more strain specific than rats of other mutant strains tested.

None of the rats of mutant strains were particularly susceptible to the growth of grafts from tumors that arose in rats of other strains. However, grafts grew in some rats of all strains tested, except in curly, and they grew in a greater proportion of cinnamons (65.8 per cent) and of blacks (48.3 per cent) than in rats of other strains.

The results obtained in this and in the previous experiments indicate that rats of the mutant strains tested, especially curly, are more strain specific than are rats of the gray Norway and inbred albino strains. To what these differences are due has yet to be determined.

E. Discussion

Because of marked differences between mice and rats in regard to metabolic processes (odor, urine, et cetera), and to the greater development of spontaneous tumors in mice (some strains develop 80 per cent mammary gland carcinomata), a comparison of the results obtained in our studies on rats and those published by investigators using mice is deferred until later. However, one point that should be mentioned is that all mice injected with carcinogenic agents survived and developed sarcomata regardless of their strain (Lewis and Lichtenstein, '39). From our studies it seems evident that, if proper technique is used in the injection of the carcinogenic agent and proper care given the treated animals, they would live until tumors induced in them developed to a transplantable size. The necessity of computing percentages from the animals that survived for 60 to 90 days only would thus be eliminated.

The outstanding work of Dunning, Curtis, and Wood ('40) and that of Dunning, Curtis, and Eisen ('40) on rats, are difficult to analyze in view of the marked differences in their results from those

described in this paper. Many of their treated rats died, and not all of those that survived developed tumors. In our investigations every one of the injected rats lived and developed a sarcoma at the injection site. Rats that received one injection of a carcinogenic agent developed tumors in as short a period of time as did rats used by other investigators which received several injections of the carcinogenic agent which, it was thought at that time, accelerated the development of tumors in the treated animals.

The results obtained by Eisen ('42) on mammary gland adenomata need not be discussed here as during the four years of our studies only two mammary gland tumors occurred in King inbred albino rats although many hundreds of these rats were reared during this time. These two spontaneous adenomata were 100 per cent transplantable into rats of their strain of origin. Growth of grafts of these tumors was slow, requiring from two to three months to attain the standard size (50 x 25 x 20 mm.) used for transplantation. These adenomata grew equally rapidly in male and in female rats. They contained an abundance of milk when they grew in a female that was nursing her offsprings. Dunning, Curtis, and Wood noted that while female rats of the seven strains they used developed many adenomata only about one in 400 spontaneous mammary gland tumors was malignant.

Selbie ('36) and also Roffo ('20) claim to have obtained susceptible strains of rats through breeding. Selbie inbred descendants of rats from which large tumors had been removed by operation. Although these rats were inbred for four years, they never became 100 per cent susceptible to the growth of grafts of the four tumors used. All of the tumors used arose in rats of unknown inheritance: they were the Jensen rat sarcoma, the Walker carcinoma 256, the Guerin rat carcinoma, and the Willis rat carcinoma.

Roffo ('20) bred a susceptible and a resistant strain of rats. The 28 tumors that occurred in the susceptible strain were 95 to 100 per cent transplantable into rats of this strain, but only five per cent were transplantable into rats of the resistant strain. On crossing the two strains, Roffo obtained only 15 per cent of takes instead of the 95 to 100 per cent to be expected from the results of our experiments on crosses between susceptible inbred albinos and resistant gray Norways. When Roffo inbred rats of the F_1 cross between his two strains, he obtained a higher percentage of takes instead of a lower one which

would be anticipated in the F_2 cross, since in later generations there would be fewer rats with the susceptible quality.

F. Summary

Litters from 14 strains, comprising 9,781 rats were used in these studies on the inducement and transplantability of tumors in rats.

Carcinogenic agents used for tumor inducement were 1:2:5:6-dibenzanthracene, 2:3-benzpyrene and methylcholanthrene. Tumors induced by dibenzanthracene required a longer time for development to the standard size than did those induced by other agents.

Every one of the rats in 30 litters from 14 strains injected with a carcinogenic agent survived and developed a tumor at the injection site.

Microscopical preparations showed that the tumors were all sarcomata.

Induced tumors developed more slowly in gray Norway rats than in rats of other strains.

Induced tumors developed earlier in males than in females of the litters used in these studies.

Tumors developed more rapidly in rats fed on a balanced diet than in rats fed on Purina fox chow.

Grafts of all primary tumors, when implanted into susceptible rats, developed to the standard size in a shorter period of time than that required for the inducement of the primary tumor.

All induced tumors were more transplantable in rats of their strain of origin than in rats of other strains.

Grafts of sarcomata that originated in the strictly inbred King *A* albino rats were 100 per cent transplantable in rats of the same strain, in rats of the F_1 cross between gray Norway and inbred albinos, and in the F_1 backcross to inbred albinos, but not in rats of other strains.

Tumors that originated in rats of less inbred strains, such as gray Norways, hybrid rats, and rats of mutant strains, either failed to grow or grew in only a proportion of the rats into which they were ingrafted.

When transplanted some of the sarcomata induced in King *A* inbred albino rats proved to be strain specific. While the grafts grew in all of the King *A* ingrafted rats, they failed to grow in rats of

other strains. Other tumors induced in King *A* rats were less strain specific. Their grafts grew not only in the King *A* rats, but also in some rats of many other strains.

Grafts of tumors that arose in New Haven albinos grew in a larger proportion of rats into which they were transplanted than did the grafts from sarcomata induced in rats of other mutant strains.

Tumor grafts grew faster when implanted into young rats than into old rats.

Growth of sarcomata grafts was not influenced by the coat color of the host into which they were implanted.

Rats of the curly strain proved to be the most resistant to the growth of grafts of sarcomata from rats of their own and of other strains.

Grafts of sarcomata induced in gray Norway rats, in hybrid rats, and in rats of mutant strains failed to grow in a large proportion of the rats of their own and of other strains in which they were implanted.

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THE EFFECTS OF CARCINOGENS ON GROWTH OF FUNGUS HYPHAE*

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A. INTRODUCTION

Mushrooms are often severely deformed by volatile impurities in crude kerosene, or in natural gas. The distortion is of the nature of an outgrowth from the smooth pileus, and may be almost the diameter of the mushroom. The upper surface is finely corrugated, often pinkish in color. The condition, known as Rose-comb, has been described by E. B. Lambert of the U. S. D. A. As yet, the specific agents responsible have not been determined.

The nature of the outgrowth and its association with crude petroleum led to the idea that the condition might be comparable to cancer. If so, a carcinogen might be expected to have some effect upon the fungus growth. The literature dealing with the effects on lower organisms is limited. *Morphological abnormalities* have been reported in paramecia and in yeast (Dodge, 1937; Bauch, 1942), though some of the work could not be repeated. *Population increases* have been noted in yeast (Dodge, *et al.*, 1941; Cook, *et al.*, 1939), and in bacteria (Goldstein, 1937; Spencer and Melroy, 1940; Hopper and Clapp, 1939). Proliferation was increased in *Obelia geniculata* by 1:2:5:6-dibenzanthracene (Reimann and Hammett, 1935) and new growth from anlagen by methylcholanthrene (Hammett and Reimann, 1935). *An increase in the life span* of a species of paramecia due to continued action of carcinogen has been reported (Spencer and Melroy, 1941), though the possibility that a mutant was formed must not be overlooked. Similar treatment of a bacterium (Spencer and Melroy, 1940) failed to produce any morphological or physiological differences after 240 transfers. An *increase in weight* has been de-

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scribed (Wright and Anderson, 1938) for the growth of *Fusarium lini*. In this single case, no effect could be obtained by means of the insoluble 1:2:5:6-dibenzanthracene, but a brief ultraviolet irradiation of a benzene solution of the carcinogen gave a water soluble substance having the stimulating action. Though the authors claim to be using the method of Boyland, their treatment is not nearly so severe. There is no certainty that the compound formed has any carcinogenic action. Boyland failed to obtain tumors with his water soluble compound. Weight measurements on *yeast* with and without carcinogen failed to show any significant differences (Dodge, *et al.*, 1937, 1941).

B. Material and Methods

1. *Inoculum*

a. Spore Germ Tubes. A heavy inoculation of spores (strain 122 J. W. Sinden) was made into 1 ml. of staling solution kindly supplied by Dr. Sinden. The suspension, with occasional shaking, was incubated at room temperature for 48 hours, then diluted to 4 ml. with sterile distilled water. Further dilutions with *DBA* and potato broth were made to give the required concentrations of chemical and spores.

b. Hyphal fragments (1) *Mushroom* (strain 110 J. W. Sinden) was grown on potato broth in a small test tube for 10 days. The mat formed was removed and ground with sterile sand. Water was added to form a suspension, and dilutions made as above.

Chaetomium sp. was grown 5 days on potato broth agar in a petri plate. One sq. cm. of agar with mycelium was taken from the colony edge and ground with sand. There were no perithecia in this area. Further treatment as above.

2. *Agar and broth suspension of DBA*

a. Containing acetone. A stock solution of *DBA* in acetone was made at a concentration of 1 mgm./ml. The appropriate amount of stock solution was added to 50 ml. of potato broth, and boiled 30 minutes (with replacement of water) to remove the acetone. This gave a hazy suspension at higher concentration which was, however, quite stable. For the agar, the *DBA*-Acetone was made up in water at twice the required strength, the acetone boiled off, and the suspension added to the double strength agar.

TABLE 1

MUSHROOM GERM TUBE GROWTH IN DECREASING CONCENTRATIONS OF 1:2:5:6-DIBENZANTHRACENE
(J. W. Sinden Strain 122)

Exp. No.		*DBA* 1/1000 x Broth	Agar	No. Sps.	Av. Init. L	Av. Incr.	Av. New Br.	T/C %	Corrected values* No. Sps.	Av. Init. L	Av. Incr.	T/C %
S7	Test	50	50	63	6.3	13.1	4.1					
	Cont.			36	6.6	16.6	4.7	79				79
S7	Test	50	50	50	5.4	14.8	3.0					
	Cont.			61	5.3	17.3	3.5	86				86
S2	Test	75	50	33	3.4	10.8	3.2					
	Cont.			52	3.3	12.3	3.2	88				88
S3	Test	200	250	65	6.4	14.7	5.5		65	6.4	14.7	
	Cont.			81	7.5	13.8	5.9	107	73	6.8	13.5	109
S5	Test	250	250	43	4.7	15.1	3.9		37	5.3	15.8	
	Cont.			42	5.4	15.2	6.1	99	42	5.4	15.2	104
S5	Test	250	250	55	5.3	15.3	5.0		55	5.3	15.3	
	Cont.			52	4.8	14.7	5.1	104	46	5.1	15.5	99
S6	Test	400	400	31	6.0	23.9	3.5					
	Cont.			46	5.7	14.5	5.0	165				165
S6	Test	400	400	56	6.1	27.3	4.2		56	6.1	27.3	
	Cont.			58	5.6	14.3	5.4	191	51	6.1	14.8	184
S8	Test	400	400	57	6.4	14.8	5.4					
	Cont.			49	6.0	14.3	5.4	103				103
S8	Test	400	400	66	5.7	13.8	5.0					
	Cont.			60	6.0	14.9	6.1	93				93
S17	Test	400	400	57	4.6	15.1	5.7		54	4.3	14.8	
	Cont.			34	4.4	13.4	6.3	113	34	4.4	13.4	110

Sps—Spores; L—Length; Br—Branches.
*Correction was made in order that the original average lengths would be more nearly equal.

TABLE 2

MUSHROOM GERM TUBE GROWTH IN DECREASING CONCENTRATIONS OF 1:2:5:6-DIBENZANTHRACENE—9:10 ENDOSUCCINATE

(J. W. Sinden Strain 122)

Exp. No.		*DBA* 1/1000 .x Broth Agar	No. tubes	Av. Int. L	Av. Incr.	Av. New Br.	T/C %	Corrected values: No. tubes	Init. L	Av. Incr.	T/C %
S12	Test	50	69	4.7	15.7	—		60	5.2	16.6	
	Cont.		62	6.1	26.0	—	60	57	5.4	24.6	67.5
S12	Test	50	63	6.2	21.1	—					
	Cont.		62	6.1	26.4	—	80				80
S14	Test	200	80	4.3	15.9	—		80	4.3	15.9	
	Cont.		81	3.9	13.4	—	119	70	4.2	13.5	118
S14	Test	200	71	5.8	19.0	6.7					
	Cont.		68	5.6	17.4	7.5	109				109
S16	Test	200	46	3.4	9.3	—		44	3.5	9.5	
	Cont.		54	4.0	11.5	—	81	50	3.8	11.6	82
S16	Test	200	63	3.3	9.5	3.9					
	Cont.		66	3.5	11.0	3.9	86				86
S13	Test	300	54	5.8	21.9	—					
	Cont.		68	5.7	23.8	—	92				92
S13	Test	300	68	6.7	24.4	—					
	Cont.		69	6.8	25.8	—	95				95
S11	Test	400	20	9.3	21.3	5.9		20	9.3	21.3	
	Cont.		31	7.4	18.1	5.7	116	25	8.8	20.5	104
S15	Test	400	80	5.9	21.5	—		73	6.3	21.9	
	Cont.		78	6.5	26.5	—	81	76	6.3	25.7	85
S15	Test	400	76	5.5	21.6	6.7		67	5.8	21.4	
	Cont.		70	5.9	24.5	6.8	88	70	5.9	24.5	87

L—Length; Br.—Branches.

Controls were made similarly with acetone, but without the *DBA*.

b. Water soluble carcinogen. (1:2:5:6-*DBA* 9-10 endo-succinate, supplied by Dr. Robert Briggs of The Lankenau Institute). Fifteen mgm. of the water-soluble carcinogen was placed in a dark glass-stoppered bottle. Two ml. of 0.06N NaOH were added and left to stand overnight. Eight ml. of water were then added and complete solution took place. Further dilution to 1:10,000 was made. The control using NaOH but minus the *DBA* was prepared at the same time.

Proper amounts of the above were used in agar and in broth. Sterilization was at 15 lbs. for 30 minutes. In the 1/25,000 broth this resulted in a haziness, perhaps due to a reconversion of the soluble carcinogen to insoluble form.

3. *Experiment*

a. The agar, containing carcinogen (or control), was melted in boiling water, and a layer about 1/8" thick placed on sterile slides in sterile petri dishes.

b. The *DBA* suspension of spores was made up to the desired concentration.

c. Crosses were made in the agar (on slides) as reference points.

d. A drop of spore suspension was placed on each cross.

e. Several drops of sterile water were placed on filter paper in the bottom of each petri dish so that each dish acted as a moist chamber.

f. The dishes were incubated for 24 hours to dry the drops.

g. Drawings of each germ tube, and measurements were made, and the whole incubated for another 24 hours at 25° C.

h. Remeasurements were made. Figures are micrometer spaces.

i. Controls without *DBA* were similarly set up and treated simultaneously.

Modifications in the above procedure were few. For Chaetomium hyphae, the time of incubation was reduced to 6 hours because of the rapidity of growth.

C. Results and Discussion

1. *Hyphal Fragments*

a. Mushroom (strain 110 J. W. Sinden). There is a marked

trend from toxicity at 1:50,000, through no effect at 1:125,000 to possible stimulation at 1:250,000 (Table 3).

TABLE 3

MUSHROOM HYPHAE GROWTH IN DECREASING CONCENTRATIONS OF 1:2:5:6-DIBENZANTHRACENE
(J. W. Sinden Strain 110)

Exp. No.		*DBA* 1/1000 x Broth	Agar	No. Hyp.	Av. Init. L	Av. Incr.	Av. New Br.	T/C %	Duration
M1	Test	133	100	52	11.1	16.1	2.4		
	Cont.			48	15.8	21.2	3.5	76	24 hrs.
M2	Test	135	100	44	15.9	31.7	4.1		
	Cont.			45	16.3	33.9	4.0	93	24 hrs.
M3	Test	75	50	38	13.4	18.0	2.4		
	Cont.			51	16.8	22.1	3.5	81	24 hrs.
M5	Test	125	125	35	15.4	8.7	0.9		
	Cont.			24	14.8	8.7	0.5	100	12 hrs.
M5	Test	125	125	61	14.0	7.2	1.2		
	Cont.			28	14.4	7.4	1.1	97	12 hrs.
M4	Test	375	250	31	12.5	7.8	0.6		
	Cont.			37	13.1	6.8	0.5	115	12 hrs.
M4	Test	375	250	47	14.8	9.4	1.05		
	Cont.			52	12.0	7.6	1.0	124	12 hrs.

Hyp.—Hyphae; L—Length; Br.—Branches.
Experiments M1 and M2 contained a small amount of gelatin. This was omitted from all later tests.

b. Chaetomium sp.: This fungus had as a great advantage its speed of growth, results being attainable in six hours. In both concentrations tested, the carcinogen reduced the growth rate, the reduction being greater at the higher concentration. Comparison with results on mushroom hyphae indicate agreement at 1:50,000, and in trend on dilution, but the faster growing Chaetomium appears to be more sensitive to carcinogen toxicity than the mushroom (Table 4).

Experiment K3b was included because the early experiments had two concentrations of carcinogen, that in the agar and that in the suspension. The results indicate that the effect is to be correlated with the higher concentration of the carcinogen in the agar.

Since these hyphal experiments were in agreement with the first experiments carried on with germ tubes, and because they were much

TABLE 4

GROWTH OF CHAETOMIUM MYCELIUM IN DECREASING CONCENTRATIONS OF 1:2:5:6-DIBENZANTHRACENE

Exp. No.		*DBA* 1/1000 x Broth	Agar	No. Hyph.	Av. Init. L	Av. Incr.	Av. New Br.	T/C %	Duration
K2	Test	75	50	1	(only 2% growing, compared to 50% in control)				
	Cont.			21	10.4	——	5.6	—	6 hrs.
K3	Test	75	50	30	9.9	15.5	0.8		
	Cont.			23	10.9	29.1	1.3	53	6½ hrs.
K3a	Test	250	250	36	14.1	26.7	1.8		
	Cont.			41	14.0	36.4	2.4	73	6½ hrs.
K3b	Test	250	50	20	13.0	18.7	1.7		
	Cont.			30	13.4	37.1	2.2	50	6½ hrs.

Hyph.—Hyphae; L—Length; Br.—Branches.

more difficult to carry through, they were discontinued. The difficulty is that only about 25 per cent of the fragments grow, whereas, with germ tubes, practically all continue growth.

2. *Germ Tubes*

At the time of discontinuing the above, the spore trials had been completed through S7. The trend was the same as with the hyphae, but the effective concentrations differed (Table 5).

TABLE 5

RELATIVE INCREASE OF TESTS IN TERMS OF CONTROLS

	1:50,000	1:125,000	1:250,000	1:400,000
Chaetomium hyphae	53		73	
Mushroom hyphae	81	98.5	120	
Mushroom germ tubes	82.5		103	178

The germ tubes appearing most satisfactory, additional work was concentrated on these. Three further experiments at 1:400,000, however, failed to show any stimulation whatever. At this time, the water soluble carcinogen, 1:2:5:6-*DBA* 9-10 endo-succinate, became available, and work was continued with it, in order to eliminate any possible effects of acetone in the previous cases (Tables 1 and 2).

The inhibition at 1:50,000 is in agreement with the earlier results, but the inability to obtain reproducible results at the lower concentrations makes it impossible to reach any conclusion.

An experiment at 1:200,000, continued for a second 24 hours, shows clearly an increasing toxicity. The ratio, test to control, dropped from 83.5 for the first 24-hour period to 48.5 for the second 24-hour period. This experiment also confirms an observation made quite early in the course of these experiments, that the rate of elongation depends upon the original length (Table 6).

TABLE 6

		0-24 hrs.		24-48 hrs.		0-24 hrs.	24-48 hrs.
		Initial Length	Rate spaces/hr.	Initial length	Rate spaces/hr.	Increase Initial length	
T	1:200,000	3.4	.39	12.7	1.46	2.7	2.0
C		4.0	.48	15.5	2.22	2.9	3.4
T	1:200,000	3.3	.40	12.8	1.03	2.9	1.9
C		3.5	.46	14.5	2.06	3.1	3.4

Pausing for a moment on the implications of this single experiment leads one to consider whether changes in techniques would not have influenced the results considerably. Certainly the degree of *difference* between test and control reaches a maximum at a given moment. In our test, the difference between growth rate of the test and that of the control increased markedly from the 24-hour reading to the 48-hour reading and may be assumed to increase even more (Figure 1). Unfortunately, by that time, the colonies have grown so large and have become so intertwined as to make measurement impossible.

The rate of elongation of fungus colonies becomes constant after the initial log phase. For the mushroom, under the experimental conditions, it may be estimated at about 13 spaces per hour. From this figure and those given above, it is possible to construct a rough graph showing the normal development. Unfortunately, we have no data on whether the linear rate would be the same for the soluble *DBA*, though reached at a later time. The work of Dodge *et al.* (1937:1941), and of Wright and Anderson (1938) support the view that the linear rate of the Test is *above* that of the Control. Both are in agreement also on the initial inhibition. It is thus obvious that one and the same concentration of carcinogen can be both inhibitory and stimulatory, the action being dependent on the time of measurement.

The present work confirms the inhibitory action of carcinogens on the early phases of growth, and shows a direct correlation between

Growth Curves of Treated and Untreated Germ Tubes

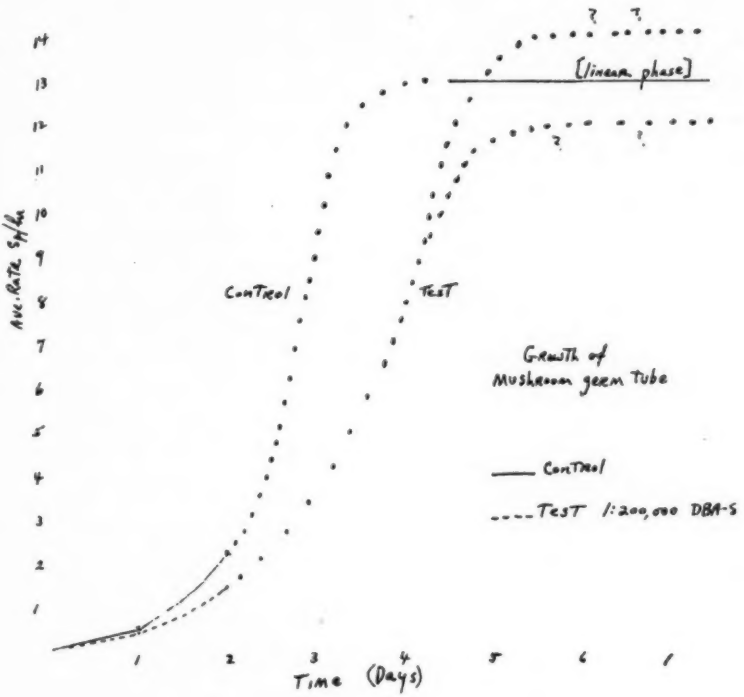

FIGURE 1

concentration and degree of inhibition. It emphasizes the importance of a continuous measurement of growth through the log and early linear phases. As to observations incapable of repetition,—Dodge's giant yeast cells; and our experiment of marked stimulation at low concentration—two explanations are possible. Either an extraneous factor was responsible, or some slight modification of carcinogen or medium during that particular experiment was sufficient to bring about the results observed.

D. Conclusions

1. During the log phase of growth of fungus hyphae, the carcinogen 1:2:5:6-dibenzanthracene,—at 1:50,000 is effective in reducing the rate of increase of growth.

2. The reduction in rate is greater on the faster growing fungus (Chaetomium).

3. Time of measurement is of utmost importance in demonstrating differences. Second day results (mushroom) showed far greater inhibition than first day results. It is probable that the greater differences found in Chaetomium may be due to its relative position on the growth curve.

4. Though stimulation, observed at 1:400,000 in the early experiments could not be verified, it is not impossible that *DBA* may under certain circumstances be stimulative as well as retardative of germ-tube and hyphal growth. The question is not answered by these data however.

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THE RELATION OF CELL DIVISION TO GROWTH RATE IN CUCURBIT FRUITS

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The interrelations between an organism and the cells of which it is composed have long been one of the outstanding problems of biology. Ever since the discovery that protoplasm occurs most commonly in those small unitary masses which are called cells or protoplasts, many biologists have regarded the organism as a cell state and have sought to explain its structure and behavior as due to the interaction of semi-independent cellular units. Others look upon larger units, the organs or entire organisms, as the true individuals, both morphologically and physiologically, and regard their division into cells as simply a necessary expression of protoplasmic differentiation. The literature dealing with this problem is extensive, but much of it has been rather speculative in character since there are not many methods by which it can be directly attacked.

One of the best of these methods, but one which has not been very widely used, is to compare the cellular composition of an organic structure during the course of its development with the growth of the structure itself. Does the structure grow, we may ask, as though it were dependent upon cellular changes within it or as though it were a single independent entity? Good material for a study of this question is provided by certain plant organs, notably ovaries and fruits, which are relatively simple morphologically and histologically, and in which growth can be easily measured over the whole developmental period. The fruits of the cucurbit family and notably the species *Cucurbita Pepo*, which includes the squashes, pumpkins, and some of the gourds, are especially favorable for this purpose.

Several years ago the writer (1) made a developmental analysis of the fruits of a number of inbred lines in this species. At all stages from young ovary primordia about 1 millimeter in diameter to mature fruits, cell diameter was measured in the epidermis, in the outer,

middle, and inner layer of the thick wall or pericarp, and in the fundamental tissue of the central placental region. In all regions during the earlier stages, the diameter of the ovary was found to increase faster than the average cell diameter, thus showing that the cells were increasing in number and that cell division must therefore be occurring. At a given ovary size, the average cell size for a given region began to increase at the same rate as the entire ovary, thus indicating that cell division had ceased in that region and that cell enlargement by vacuolation was taking place. This continued until final fruit size was reached or until (in the inner regions) the tissues collapsed. In each region the cellular history was the same—first a period of cell division which ceased at a given ovary size, followed by a period of cell enlargement which sometimes resulted in a volume nearly a million times as large as that of the early primordial cells. The point at which cell division ceased was determined not only from the relation of cell size to ovary size but from direct observation.

In every case division ceased first in the placental region and then in the successively outer layers until in the epidermis it persisted almost to the end of growth. In most regions the average cell size increased considerably during the period of cell multiplication, indicating that in each successive division the size attained by a cell about to divide was greater than in the preceding one. This increase was always greatest in the innermost regions and less in the progressively outer ones.

The cells measured in every case (except the epidermis) were those of the fundamental parenchyma which constitutes the great bulk of the ovary. Vascular bundles, ovules, and other specialized structures were not considered. Even in this simple parenchymatous tissue, however, developmental analysis thus shows a rather complex picture of differences in cell size and activity.

This analysis was in terms of increasing organ size, regardless of rate. For six seasons the writer has now measured the growth rate of the developing fruit in a number of inbred lines and thus determined the course of size increase for the organ as a whole (2). This was done by making daily caliper measurements of ovary dimensions and calculating volumes from these. In every case the first part of the growth period seems to be exponential in character, the increase in

volume each day being a constant percentage of the volume already attained. When the logarithm of volume is plotted against time an essentially straight line therefore results for this part of the growth period. In time, however, the rate of growth becomes gradually less until it finally ceases as full size is reached. The logarithmic growth curve thus has the general character of those shown in Figure 1, an

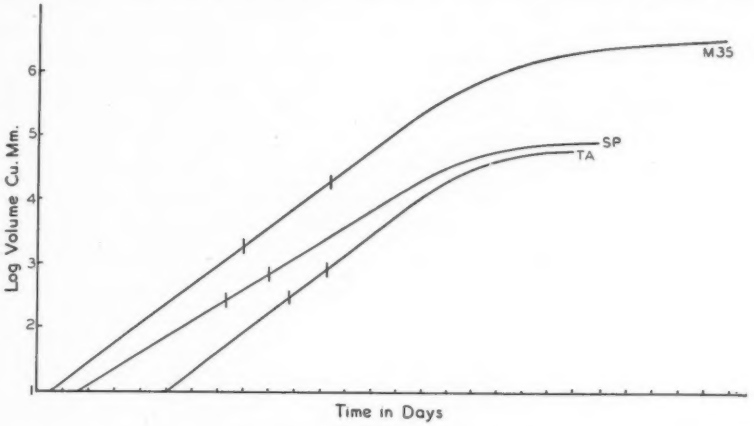

FIGURE 1

GROWTH CURVES OF FRUITS OF THREE LINES OF GOURDS, FROM LOGARITHMS OF VOLUME PLOTTED AGAINST TIME

In portion to the left of vertical bars, growth is by cell division; in portion to the right, by cell enlargement. Portion between bars is period during which division ceases, beginning at central region and ending with middle wall layer.

ascending straight line followed by a gradually flattening curve. All parts of this curve are of greater duration in large-fruited types like *M35* than in small ones like *TA*, and final size is thus primarily a function of duration of growth rather than of its rate. Differences in rate do occur between lines, however, as between line *SP* and the other two shown in Figure 1.

By combining the data from these two investigations it is therefore possible to determine the point on the growth curve at which, for any region of the ovary, cell division ceases. The published data (1, Table 1 and Figures 1-9) give the average cell diameters at which division ceases, and from the graphs the ovary diameters at these stages can

be determined. By finding the ovary volume corresponding to each diameter, the points on the growth curve where cell division ceases can then be determined.

The growth curves shown in Figure 1 are from the author's published data for these lines of *Cucurbita Pepo* (2). In each curve the points where cell division ceases in the placental region (left) and in the middle wall (right) are indicated by vertical bars. The data are for three lines described in the 1939 paper. Previous to the point indicated by the left bar, cell division is occurring in all regions. After the right bar, practically all growth is by cell expansion, since cell division persists now only in the epidermis and the very narrow outer wall which are almost negligible in the volume of the fruit as a whole. Between the two bars is the period (rather short in small-fruited types, somewhat longer in larger ones) when the basis of growth changes from an increase in *number* of cells to an increase in their *size*. A notable fact which is evident from these graphs is that this fundamental alteration in the cellular basis of ovary volume has no visible effect whatever on the steady progress of growth in the organ as a whole. If the growth of an organ depended on the behavior of its constituent cells, one might expect that at this critical point in the life of those cells which comprise the great bulk of it there would be a change in the growth rate of the organ as a whole. Such is not the case. Throughout the entire developmental process which, as has been shown, involves a good deal of change in the size and activity of cells, the growth of the ovary as a whole marches on at a constant pace. If simplicity of behavior is the criterion of organic individuality, this is manifest more clearly by the ovary as a whole than by any of the cells which compose it. These facts were briefly mentioned in a general discussion of the cell-organ relationship by the writer several years ago (3).

This conclusion is further strengthened by a study of the increase in dry weight as the ovary grows. In line *TA*, 43 young ovaries were collected ranging in diameter from 3 to 20 mm. These were measured and their volumes calculated in the same way as before. For each, the dry weight was now determined. The logarithm of the dry weight of each ovary, in milligrams, was plotted directly below its corresponding ovary volume on an average growth curve for line *TA* (Figure 2). It is evident that these dry weights increase at a constant

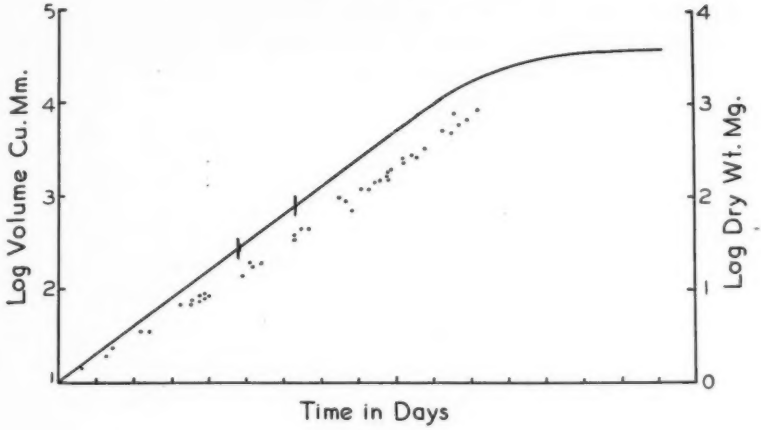

FIGURE 2

GROWTH CURVE OF LINE *TA*, AS IN FIGURE 1

Small circles below are logarithms of dry weights in mg. of ovaries the fresh volumes of which correspond to the points on the curve directly above them. (Scale at right.)

exponential rate just as do the corresponding volumes. This is the more noteworthy since after cell division ceases, increase in the size of each cell seems to be due chiefly to an expansion of its vacuole. Evidently the amount of material, exclusive of water, from which the ovary is built, enters it at a constant exponential rate regardless of whether the fundamental tissue cells are rapidly vacuolating or not. After vacuolation begins, more of the plastic material is probably built into cell walls and into the tissues of the developing ovules.

The steadily increasing water content of the tissue during the exponential growth period is shown by the fact that dry weight grows less rapidly than ovary volume. During later growth, of course, when cells of the fundamental tissue are being packed with food, when their walls are thickening, and when the seeds are becoming mature, dry weight increases much more rapidly than volume. This portion of the dry weight curve is not shown in Figure 2.

DISCUSSION

The fact that in the first part of the growth period the material of which the ovary is built (whether measured by total volume or by

dry weight) enters it at a constant exponential rate, regardless of what its constituent cells are doing, suggests that there is a relatively simple control of the growth process. This allocates a definite and regularly increasing amount of plastic material to each ovary, an amount which seems to have no relation to whether it is being distributed among many cells or few, or, indeed to any other changes (such as anthesis) which may be going on in the developing organ. This simplicity and constancy of its over-all growth behavior is evidence that the biological individual here chiefly concerned is the organ as a whole rather than each or any of the cells which compose it. Just as the form of the developing organ progresses steadily through a simple series of changes even though its cells are dividing in all planes and present a chaotic picture to the observer, so the size of the organ also progresses steadily regardless of cellular changes within it. The idea that the organ rather than its constituent cells is the dominant entity in development is in harmony with a good deal of evidence from other sources. What are the mechanisms by which this many-celled individual controls its development in size and form are not known.

Summary

By combining data from a developmental analysis in terms of cell size with data from measurement of actual growth rates in the fruits of several inbred lines of *Cucurbita Pepo,* it is possible to determine where growth chiefly by cell multiplication ceases, and where growth chiefly by cell expansion begins. Through all this early developmental period growth proceeds at a constant exponential rate. At the point when cell division ceases and rapid vacuolation begins, there is no change in this rate. Increase in dry weight shows the same constant progression regardless of cellular changes. It is concluded that the dominant biological individual here concerned is the organ as a whole rather than any of the cells which compose it.

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A MODIFIED HELIANTHUS TEST: I. COMPARATIVE ELONGATION RATES EMPLOYING TWO METHODS OF APPLYING THE GROWTH FACTORS

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A. INTRODUCTION

The purpose of this paper is to describe a new technique for determining the effect of cell-elongating growth substances on *Helianthus annuus* seedlings. The new method is a modified technique in which the diffusion rate of the growth-promoting substance is increased by directly applying the material to be tested to the cut surface of the test plants. This is contrasted with the agar block method in which the growth-promoting substance is first incorporated in the block and then placed on the cut surface. In these preliminary experiments the capillary tube method has been found practical for testing various solutions known to affect cell-elongation. This paper is the first in a series of related papers concerned with the modified *Helianthus* test.

It is well known that recent investigations in plant physiology have been concerned with such growth-promoting substances as auxin *a*, auxin *b*, and heteroauxin. Various methods for testing the effect of growth-promoting substances by measuring cell elongation have been employed since the initiation of the *Avena* coleoptile test by Boysen-Jensen (7, 8, 9) and by Páal (16). This test has been modified by various workers such as Cholodny (11), Söding (19), Haagen-Smit and Went (14) and others as mentioned by Thimann and Went (21). Investigations have been made with plants other than *Avena sativa*. Among these to be noted are: the *Raphanus* test of Van Overbeek (15), the *Lupinus* test of Dijkman (12), and various pea tests as listed by Söding (19). Fliry (13) and others, as well as Beck and Donnelly (2) have worked out techniques employing *Helianthus annuus* as the test plant.

The *Helianthus* hypocotyl with its distinct zone of cell elongation lends itself well to a study of the effect of auxins which are specific for cell elongation. *Helianthus annuus* seedlings have been used in these laboratories to study growth by cell elongation (1, 2, 3, 4). A normal condition of the cells in the hypocotyl has been established by a detailed analysis of cell enlargement in the epidermal and cortical cells (4). It has been shown by direct measurement that the cells of different regions of the hypocotyl are not of the same size (4). The cells near the cotyledons are considerably smaller than those further removed. It has been further shown that since growth by intussusception takes place here as a result of the metabolic activity of the protoplasm during cell enlargement, growth should be considered as occurring in this region (4). Beck and Donnelly (2), in order to induce cell enlargement, applied the usual agar block and lanolin paste impregnated with the growth-promoting substance. They measured growth and growth stimulation by means of a measuring microscope. The method they developed is known as the *Helianthus* test.

B. Methods

Large seeds of *Helianthus annuus* of the same strain and age as those employed by Beck, Schocken, and Donnelly (1) were used in these experiments. The test plants were cultured in pots filled with soil consisting of one part German peat and one part quartz sand. Both the soil and the pots were sterilized in the autoclave at 20 pounds pressure for 20 minutes. Before planting the seeds, the soil was moistened as nearly as possible to field capacity with standard Knop's nutrient solution. After the seeds were planted to a depth of 5 mm., the pots were placed in a carefully regulated dark incubator. Seedlings 90 hours old from the time of planting were used throughout these experiments.

A dark chamber or incubator (Figure 1) designed and built at these laboratories was used to keep the plants at 27°C., and at a relative humidity of 90 per cent. The galvanized sheet metal incubator is placed in a tray filled with a layer of sand which acts as a seal. The humidifier consists of a tray filled with water on which rests the plant rack supporting the potted plants. In order to remove the pots, the glass window, conveniently placed in grooves, is pushed to the right. A measuring microscope mounted on a movable carriage

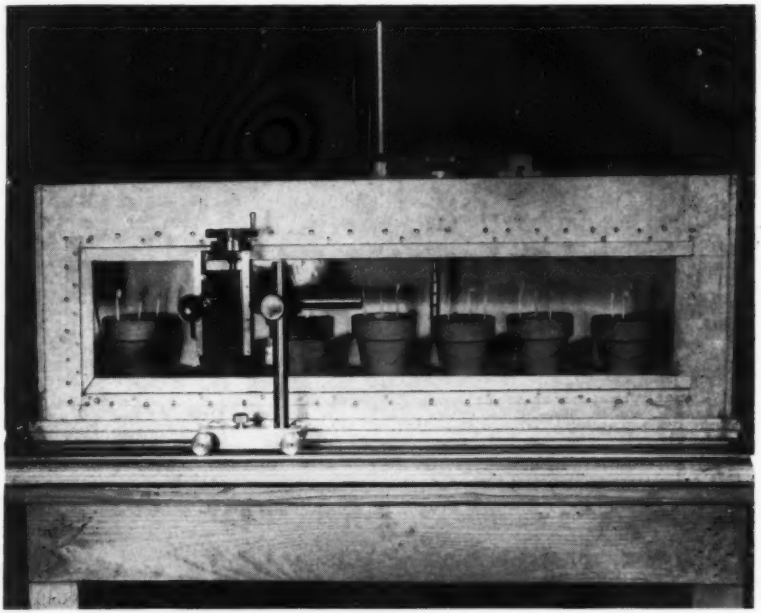

FIGURE 1

INCUBATOR AND MEASURING MICROSCOPE USED IN THE MODIFIED HELIANTHUS TEST

is used to make measurements through the glass window. All work is conducted in red light, since treated and untreated plants alike bend in white light, and auxin shows a decrease in sensitivity in light of wave lengths shorter than 55 $m\mu$ (21).

In these preliminary experiments when the seedlings were 90 hours old and approximately 4.5 cm. high (12), the cotyledons as well as the uppermost 5 mm. of the hypocotyl were removed. Individual variations were observed in many of the seedlings incubated under the above conditions. This is no doubt due, in part at least, to genetic differences. It was found that among the large number of test plants of the same age and height used during these series of experiments that the growth curves were approximately the same for each plant. From reference to previous work (1), it was accepted that the cells of the hypocotyl proliferate directly below the base of the coty-

ledons within a distance less than 2 mm., and at a distance of 35 mm. below the cotyledons the cells are mature (1). Accordingly, the seedlings were decapitated approximately 5 mm. below the base of the cotyledons in order that the zone of cell enlargement might be used for these experiments. The zone from 5 to 15 mm. below the base of the cotyledons was chosen for all work because cell enlargement has been shown by Ruge (17) and others to take place here. By actual trial, Beck and Donnelly (2) found that this zone of elongation used for testing should not be very long, since a great difference in the growth rate of the uppermost and lowermost cells introduces a considerable but indeterminate error in the numerical expression. It was found, therefore, that not more than 20 mm. should be taken for an experiment. The zone was limited by the cut surface at the top and by a hair-like needle of the plant *Bivonia stimulosa* marking the termination of the 10 mm. zone (Figure 2). A sterile thin-walled Pyrex capillary tube, approximately 3 mm. in diameter, 15 mm. in

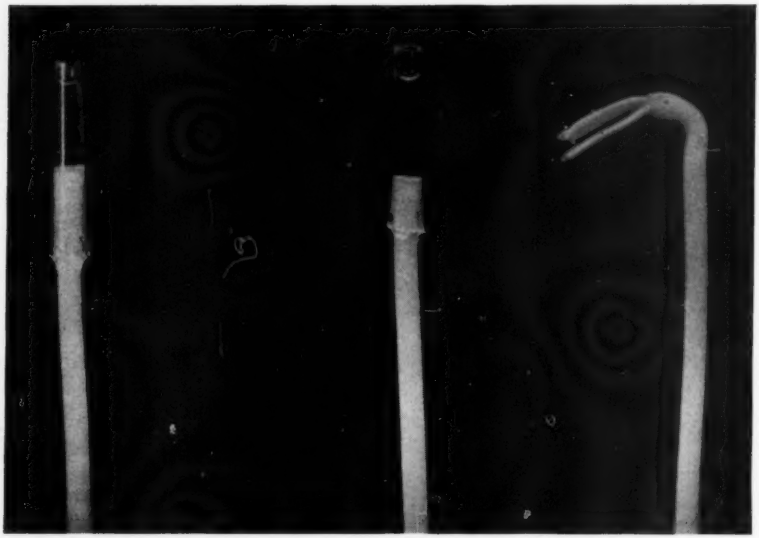

FIGURE 2

NINETY-HOUR-OLD SEEDLINGS, SHOWING ATTACHMENT OF CAPILLARIES AND METHOD OF MARKING THE 10-MM. ZONE

length, and weighing less than the excised upper portion of the seedling, was affixed over the cut surface. Commercial petrolatum was applied with a wooden applicator at the bottom of the tube to insure against leakage of the liquid placed in the tube. The tube above the cut surface was filled with the desired solution by means of a micropipette. Decapitated plants, prepared similarly to the test plants, were used as controls. McIlvaine's buffer solution (20), used as the basic medium of the test solutions, was placed in the tubes of the control plants. The seedlings were taken out of the incubator only once during one set of experiments long enough to apply the growth-promoting substance and to prepare the control plants.

Measurement of growth was made by a measuring microscope having a magnification of 32x and a range of 50 mm. It permitted measurements to be made over a range of 100 mm. and with a precision of 0.001 mm. The difference between the readings at the cut surface and at the point of insertion of the needle was the distance measured in determining the increment of growth. The initial length of this zone was made at the beginning of the experiment and subsequent readings were made at 24, 48, and 72 hour intervals. The growth-promoting substance used was heteroauxin (indole-3-acetic acid, Eastman Kodak Company) in concentration of 0.2 mg. per liter of McIlvaine's buffer solution of pH 3.6. In a subsequent paper, dealing with the effect of pH on this zone of *Helianthus annuus* the choice of this particular pH and medium will be discussed. This concentration of 0.2 mg. of indole-3-acetic acid per liter of McIlvaine's buffer solution was found to be the optimum in accordance with that concentration found most favorable by Went and Thimann (21), by Beck and Donnelly (2) and other workers.

C. Experimental

It was the purpose of these preliminary experiments to compare elongation rates in the zone of elongation of the hypocotyl of *Helianthus annuus* employing two methods of applying the growth factors. The original length of the zone of elongation used was measured from the cut surface to the inserted needle at the beginning of the experiment. The length of this original zone was taken in succeeding periods of 24, 48, and 72 hours. The per cent growth increment over the original length in each period was determined as indicated in Table 1.

TABLE 1

GROWTH IN THE ZONE OF CELL ENLARGEMENT IN THE HYPOCOTYL OF *Helianthus annuus* SEEDLINGS

Agar block method	Elapse of time in hours							
12 Test Plants*	0		24		48		72	
14 Control Plants†	Test	Control	Test	Control	Test	Control	Test	Control
Average length in mm.	9.64	9.57	11.05	10.47	11.79	10.76	11.95	11.56
Limits of this average	9.16 to 10.18	8.90 to 9.97	9.78 to 12.30	9.80 to 10.99	10.68 to 13.08	10.07 to 11.08	10.91 to 13.77	10.17 to 12.31
Average % increase in length	0.00	0.00	14.66	9.56	22.33	12.49	24.07	16.54
Limits of this average	0.00	0.00	6.54 to 24.73	4.01 to 13.93	16.59 to 28.74	9.25 to 16.39	14.44 to 46.80	9.91 to 29.31
Critical ratio (D/E_D)	0.00		4.21		10.40		3.81	
Capillary method								
12 Test Plants*								
15 Control Plants†								
Average length in mm.	9.81	9.73	14.67	11.80	16.69	14.81	18.12	16.45
Limits of this average	9.47 to 10.03	9.31 to 10.42	12.25 to 17.58	11.09 to 13.80	13.88 to 18.29	13.23 to 17.18	14.91 to 20.95	14.37 to 24.27
Average % increase in length	0.00	0.00	49.15	21.75	70.11	52.30	84.66	68.11
Limits of this average	0.00	0.00	29.21 to 81.23	9.70 to 44.15	44.34 to 88.35	35.22 to 74.77	47.92 to 111.46	41.57 to 132.91
Critical ratio (D/E_D)	0.00		8.06		4.98		2.91	

*Test seedlings, 90 hours old, treated with indole-3-acetic acid 0.2 mg. per liter of McIlvaine's buffer, pH 3.6.
†Control seedlings, 90 hours old, treated with McIlvaine's buffer, pH 3.6.

A large number of plants were used, usually 25. Of this, a number of individuals had to be eliminated during the course of an experiment due to curvature, accidental mechanical injury, and the like. Of the resulting plants, only those showing 10 per cent deviation or less from the mean, during any period of growth, were taken in computing the final results. Since there was variation in each plant, due in part to genetic differences, statistical methods were employed to test the significance of the results. These are shown in Table 1 as the critical ratio which was determined by the recognized mathematical formula, D/E_D, in which the differences in the means (D) is divided by the probable error in the difference of the means (E_D). The probable error of the difference of the means of the control and test groups was calculated from:

$$E_D = 0.6745\sqrt{\frac{\sigma_1^2}{n_1 - 1} + \frac{\sigma_2^2}{n_2 - 1}}$$

There was a set of plants for each method of applying the growth factors; namely, the agar block method and the capillary tube method. There was a control group for each set prepared similarly to the test plant with McIlvaine's buffer solution (pH 3.6) incorporated in the agar block according to the methods of Beck and Donnelly (2) and used to fill the capillary tube in the second method. An analysis of this table shows that more absolute growth is obtained in the capillary method giving maximum growth within 24 hours, whereas the agar block method is more significant at the end of the 48 hour period, with growth leveling off after 48 hours as seen in the graph (Figure 3); the average starting length in the zone of elongation is nearly the same in both methods. Variations in this zone due to experimental error are compensated for in part by determining the per cent increase in length from the initial zone of each plant. The control growth in the capillary method is greater than that of the agar block containing the growth-promoting substance. In both methods, extending the period of growth to 72 hours does not give any additional information but shows a lessening of the growth process in both cases. Variation may be slightly less in the agar block method but the increase of absolute growth in the capillary method over that of the agar block technique will make errors due to variation correspondingly less. The critical ratios show that all values of

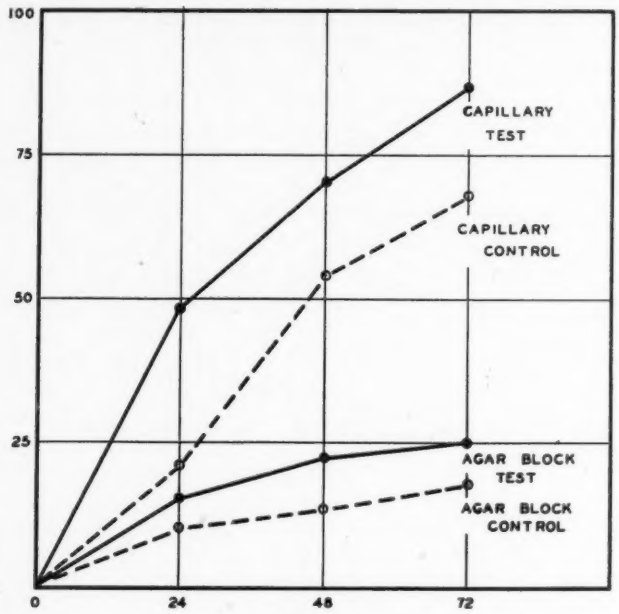

FIGURE 3

COMPARISON OF CAPILLARY TUBE AND AGAR BLOCK METHOD OF APPLYING GROWTH-PROMOTING SUBSTANCES TO *Helianthus Annuus*

The solid lines show test plants treated with 0.2 mg. Indole-3-acetic acid in a liter of McIlvaine buffer, pH 3.6. The broken lines show control plants treated with McIlvaine buffer, pH 3.6.

The abscissa is in hours, the ordinate is in percentage of increase in length of original zone.

both methods are statistically significant since according to recognized mathematical criteria a critical ratio of 2 is probably significant and one of 3 or more indicates that the difference between the averages is a significant and not a chance difference. The graph (Figure 3) clearly shows a greater response of the plant to the growth-promoting substance in the capillary tube than in the agar block. The graph also shows that in the capillary method for the first period there is an average per cent increase in length in the zone of elongation of 49.15 against that of 14.66 in the agar block; in the 48 hour period

the capillary tube method shows a growth of 70.11 per cent, the agar block 22.33 per cent; and at 72 hours the capillary technique shows an 84.66 per cent growth in contrast to the 24.07 per cent of the agar block.

D. Discussion and Conclusion

The results obtained by the modified *Helianthus* test seem to compare favorably with those obtained by other methods. As compared with the *Avena* coleoptile test employing angle determinations, the technique employed in the modified *Helianthus* test is simpler since increments in growth are measured directly. The modified *Helianthus* test provides a means of overcoming the diffusion rate difficulties which may be an important factor in such tests as the straight *Avena* test and *Helianthus* test which employ the agar block. That this is recognized may be seen by citing Went and Thimann (20), who mention that only a percentage of the heteroauxin from the agar block diffuses into the plant. In the modified *Helianthus* test this diffusion effect is minimized and may serve, therefore, as an excellent means of testing growth substances of greater molecular weight. Another variable is removed by using *Helianthus annuus* as the test plant as may be pointed out from the fact that there is no regeneration of the auxin production in the tip of the plant as there is in *Avena* and *Zea Mays* (5) where it becomes necessary to decapitate several times in order to eliminate the auxin production by the "physiological growing tip" of these monocotyledons. Another point in favor of this method is that bleeding does not interfere with the experiment while in the agar block method of Beck and Donnelly (2), it has to be carefully controlled. Moreover, as has been pointed out in Table 1, a 24-hour period is a sufficient length of time to produce maximum growth in the capillary tube method. From preliminary experiments it was found that the percentage growth obtained by this method more nearly approximated that of normal growth in the intact plants than was obtained by the agar block method. These results indicate that there is a more rapid rate of diffusion in the capillary tube method. The differential diffusion rate of the growth-promoting substance may also be a limiting factor making it difficult to determine the actual concentration of the growth promoting substance in the block.

As many variables as possible were controlled throughout the experiments. The pH effect was controlled by using a buffer solution as near as possible to the pH of the normal plant cells (18). The only factor for which no compensation was made was the food factor. The seedling when decapitated was deprived of its normal food and auxin supply. This nutrient deficiency evidently did not show up in the first 24 hours and therefore did not mask the hormonal effect of the applied heteroauxin. Besides this, the decapitation insured against any natural formation of heteroauxin and results in growth may be considered to be directly the results of the applied growth-promoting substance. The effects due to wounding (6) will be considered in a subsequent paper.

The 72-hour growth period is not a favorable one for the study of growth because of a number of factors which may affect growth such as: deficiency of food supply, deficiency of growth substance, tissue changes due to oxidation, injury and the like. Preliminary experiments indicated that even for a short period of time the capillary method was better than the agar block, but the results were more striking when carried out over a longer period of time as shown in the graph, Figure 3. Furthermore, in other preliminary experiments, little difference was seen in the comparison of the results employing agar blocks treated with McIlvaine's buffer pH 3.6, and those of untreated decapitated plants. This shows that the agar block is very inefficient over the 72 hour period.

E. Summary

1. A new type of Helianthus test is described in which the substance being tested is admitted directly in solution to the cut surface of the decapitated hypocotyl to minimize diffusion effects.

2. The importance of the rôle of diffusion rate was brought out by comparing this method with that of Beck and Donnelly (2).

3. The zone of cell elongation between 5 and 15 mm. below the cotyledons of *Helianthus annuus* seedlings, 90 hours old, 45 mm. high, proved to be an excellent indicator of growth stimulation. This verified the results of Beck and Donnelly (2).

4. This method which results in more absolute growth than the agar block method, serves as a simple, rapid, accurate way of determining the growth promoting power of certain substances on plant cell enlargement.

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A MODIFIED HELIANTHUS TEST: II. THE EFFECT OF pH IN CERTAIN MEDIA ON THE ZONE OF CELL ELONGATION IN THE HYPOCOTYL OF *HELIANTHUS ANNUUS*

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A. INTRODUCTION

In a previous paper (2) describing the modified *Helianthus* test for measuring cell elongation in *Helianthus annuus* seedlings, it was mentioned that pH of the medium is an important factor influencing this form of growth. The present paper presents evidence of the importance of pH. This fact has been recognized by such workers as Thimann and Went (10), White (11), and Gustafson (4). Strugger (8, 9) has been able by acidmetrical graded buffer mixtures to force non-growing cells to accept the colloidal state of growing cells. If this is true, then, one must not only consider the effect of media without the growth-promoting substance present, but one must also consider the hydrogen-ion effect on cell elongation. Ruge (5) has also shown that cell elongation is influenced indirectly by acidity gradients which affect the plasticity and extensibility of the cell membrane (6). Gessner (3) demonstrated that when hydrogen-ion concentration changes from pH 5 to pH 4 there is a corresponding increase in the plastic and elastic extension, and therefore more growth. This is especially true for growth by cell elongation.

These observations have led to further investigations on the effect of media, pH, and concentration of a known growth-promoter on the zone of cell elongation of *Helianthus annuus* seedlings. The results of these investigations are presented in this paper. The nutrients necessary for plant growth can be wholly or in a large part supplied by synthetic ones. This is, of course, of great practical as well as theoretical value in the study of plant growth. Many nutrient solutions have been recommended for use in plant work.

Among these, Knop's solution is well known. It was, therefore, used as the initial medium in a series of experiments employed in the modified Helianthus test (2). Other media were also tried as a means of comparison in an attempt to find that medium most favorable and at the same time most simple. No attempt was made to find a specific nutrient solution, but one which would not mask the effects of additional growth-promoting substances on the zone of cell elongation in the hypocotyl of *Helianthus annuus* seedlings.

B. Methods

The experimental procedure has already been described in detail in the modified *Helianthus* test (2). Etiolated 90-hour-old *Helianthus annuus* seedlings cultured according to the method of Beck, Schocken, and Donnelly (1) were used. For each experiment a series of plants were chosen which showed uniformity of growth and freedom from bending. Growth increments were determined with the aid of a measuring microscope at 24-, 48-, and 72-hour intervals. Percentage growth was computed for each of these periods by using the initial length of the zone of the hypocotyl selected as the zero percentage reading.

The effect of various hydrogen-ion concentrations of McIlvaine's buffer were tested and the optimum pH determined. Comparisons were also made to Knop's solution; a 12 per cent sucrose solution; and glass distilled water. The test solutions were placed in capillary tubes affixed to the decapitated hypocotyls.

C. Experimental

Results obtained in the experiments employing McIlvaine's buffer solution in pH's of 2.8, 3.2, 3.6, 4.0, and 4.5 are shown in Table 1. The results were checked by the generally accepted statistical method of determining the critical ratio by the formula, D/E_D, in which D is the difference of the means of the two experiments being compared and E_D is the probable error of the two. This is determined by the formula:

$$E_D = 0.6745\sqrt{\frac{\sigma_1^2}{n_1 - 1} + \frac{\sigma_2^2}{n_2 - 1}}.$$

An analysis of Figure 1 which is based on the data from Table 1,

TABLE 1

THE EFFECT OF DIFFERENT pH'S OF McILVAINE'S BUFFER SOLUTION ON THE GROWTH OF THE ZONE OF CELL ENLARGEMENT IN THE HYPOCOTYL OF *Helianthus annuus* SEEDLINGS

pH of solution	*No. of plants used	Average length of original zone in mm.	Averae % increase in length of zone 24 hours	48 hours	72 hours	Critical Ratio D/E_D Period of time 24	48	72
2.8	12	9.68 †(9.25-10.05)	9.48 (2.56-17.14)	12.19 (8.60-14.95)	14.22 (9.74-21.10)	6.66	17.21	12.53
3.2	11	9.77 †(9.25-10.20)	21.84 (11.80-27.46)	36.60 (22.29-49.79)	40.69 (24.41-59.31)	0.04	5.45	5.68
3.6	15	9.73 †(9.31-10.42)	21.75 (9.70-44.15)	52.30 (35.22-74.77)	68.11 (41.57-13.29)		reference solution	
4.0	12	9.71 †(9.05-10.12)	12.00 (7.44-19.11)	35.27 (26.12-51.19)	46.32 (30.80-84.50)	5.10	6.02	4.27
4.5	8	9.62 †(9.39-10.09)	9.95 (7.53-13.97)	28.89 (18.38-38.16)	34.69 (18.96-50.48)	6.38	7.96	6.39

*Seedlings, 90 hours old.
†Limits of average.

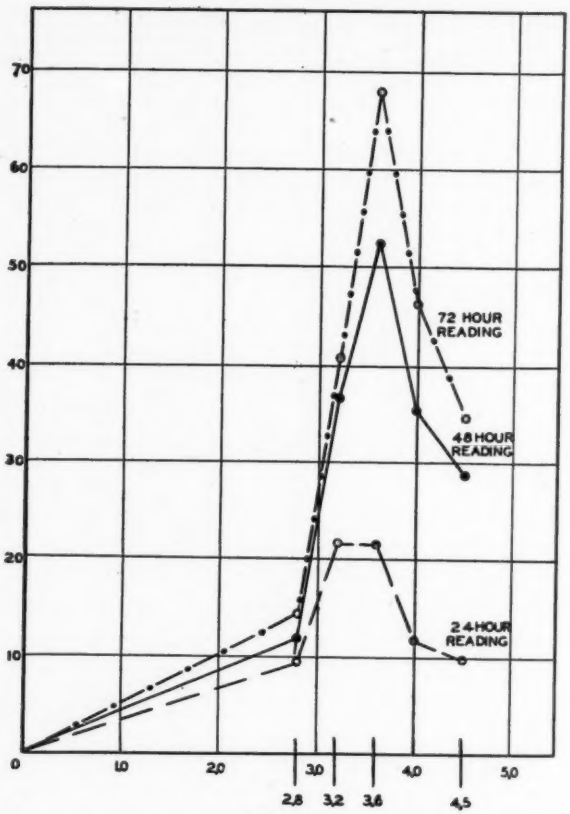

FIGURE 1

THE EFFECT OF DIFFERENT pH'S OF McILVAINE'S BUFFER SOLUTION ON THE GROWTH OF THE ZONE OF CELL ENLARGEMENT IN THE HYPOCOTYL OF *Helianthus Annuus* SEEDLINGS

(The abscissa in pH, the ordinate in increase in length over the original zone.)

shows that at the end of the 24-hour period both pH 3.2 and pH 3.6 of McIlvaine's buffer solution were found to be approximately equal in their effects but at the 48-hour period, particularly, and at the 72-hour period, although this latter is not so noticeable, the difference is significant. The critical ratios for these periods also substantiate

TABLE 2

A COMPARISON OF THE EFFECT OF VARIOUS MEDIA ON THE GROWTH OF THE ZONE OF CELL ENLARGEMENT IN THE HYPOCOTYL OF *Helianthus annuus*

		pH 4.5 12% Sucrose	pH 4.5 Knop's solution	pH 4.5 Glass Distilled H_2O	pH 4.5 McIlvaine's Buffer Solution	pH 3.6 McIlvaine's Buffer Solution
*Number of plants used		16	12	12	8	15
Average length of original zone in mm.		9.74 †(9.37-10.20)	9.61 (8.59-10.31)	9.65 (9.04-10.53)	9.62 (9.39-10.09)	9.73 (9.31-10.42)
Average % increase in length of zone	Hours					
	24	14.94 †(10.64-26.62)	15.85 (5.61-31.08)	14.90 (9.81-23.17)	9.95 (7.53-13.97)	21.75 (9.70-44.15)
	48	36.76 †(26.78-54.63)	46.49 (22.61-68.12)	33.35 (24.77-53.81)	28.89 (18.33-38.16)	52.30 (35.22-74.77)
	72	48.50 †(34.21-64.82)	59.35 (36.56-82.48)	45.96 (26.09-74.52)	34.69 (18.96-50.48)	68.11 (41.57-132.91)
Critical Ratio D/E_D	24	Reference solution	0.45	0.036	4.94	3.67
	48		2.89	1.29	3.22	7.04
	72		2.77	0.616	11.83	4.22

*Seedlings, 90 hours old.
†Limits of average.

the differences in results as being truly significant. The results were checked statistically as mentioned since here as in previous experiments out of the number of plants used for each experiment some had to be eliminated due to mechanical injury, bending, and variations partly due, no doubt, to genetic differences. Plants, therefore, deviating 10 per cent or more from the mean were excluded in the final analysis. The results show that the solution with a pH of 3.6

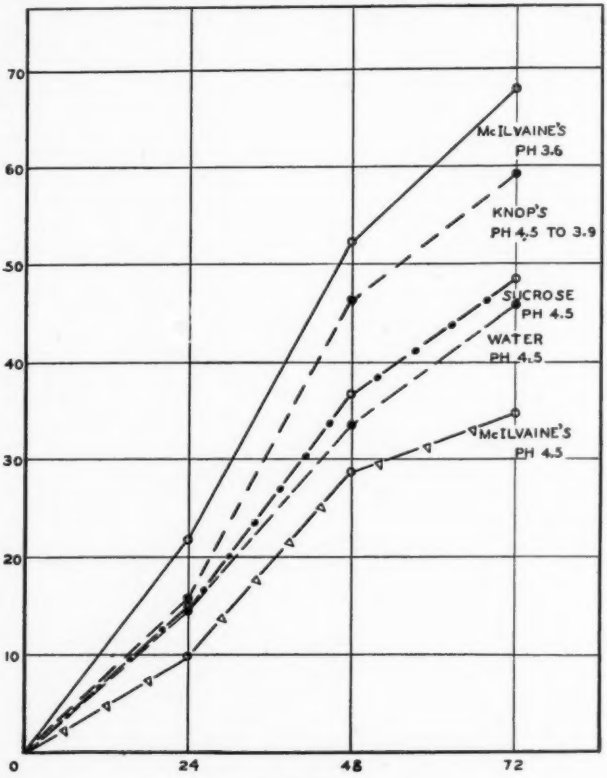

FIGURE 2

A COMPARISON OF THE EFFECT OF VARIOUS MEDIA ON THE GROWTH OF THE ZONE OF CELL ENLARGEMENT IN THE HYPOCOTYL OF *Helianthus Annuus*

(The abscissa is time in hours, the ordinate is increase in length over the original zone.)

is statistically significant at the end of the 48- and 72-hour periods. The graph (Figure 1) shows clearly, too, that a pH of 3.6 is the optimal pH for the modified *Helianthus* test.

Data in Table 2 show the results when different media of the same pH were used. Figure 2 is based on these data. From the figures in the table it can be seen that there is no significant difference between the results using 12 per cent sucrose solution, distilled water, and Knop's solution at a pH of 4.5. However, the critical ratio between the 12 per cent sucrose solution and the McIlvaine's buffer solution at 4.5 show very significant results. When McIlvaine's buffer of pH 4.5 is compared with the same solution of pH 3.6 another significant difference is noticeable.

D. Conclusions and Discussion

That pH has a definite effect on growth by cell enlargement in the hypocotyl of *Helianthus annuus* seedlings has been shown. Different pH's of the same medium show that the pH effect is important and unless accounted for, may interfere with the effect of the applied growth-promoting substances on cell elongation. This would substantiate the findings of other workers (3, 5, 7, and 8).

From the data presented it is clearly seen that McIlvaine's buffer solution is more favorable for growth at a pH of 3.6 than is Knop's solution at the same pH. It was observed during the course of the experiments that there was an increasing amount of precipitation of salts in the Knop's solution with a corresponding shift of pH toward the acid side. For this reason, Knop's solution was abandoned. This shifting of the pH may account for the results observed in Figure 2 where a comparison of Knop's solution, a 12 per cent sucrose solution, glass distilled water, and McIlvaine's buffer are compared at a pH of 4.5. The Knop's solution by experiment was found to change from a pH 4.5 at the beginning of the experiment to a pH of 3.9 after 72 hours. This solution then approached the more optimal pH of 3.6 as determined with McIlvaine's solution. This shifting of pH in the Knop's solution would also account for the results showing more growth taking place in Knop's at 4.5 than in the buffer solution of pH 4.5 which did not change in pH and which was beyond the optimum pH for this solution. A similar change of pH toward the more favorable range could have taken place in the glass

distilled water and in the sucrose solution since neither were buffered. Guttation from the cut hypocotyl into the media in the capillary tube may have been sufficient to change the pH of these two solutions to one approaching the normal pH of the tissue solution (7). The pH of the McIlvaine's buffer solution remained constant throughout the period of the experiment and no precipitation took place. These two factors made it a more favorable medium than Knop's solution for testing cell elongation in *Helianthus annuus* seedlings.

From Figure 2 it would seem that what is an optimal pH for one medium is not necessarily the optimal for another. In this case, therefore, on comparing the difference between McIlvaine's and Knop's solutions, the buffer system may be more efficient since Small (7) discovered that a phosphate buffer system plays an important rôle in the normal growth of *Helianthus annuus* seedlings. Moreover, he has shown that the hydrogen-ion concentration of injured cells is approximately 3.4. This may explain why there is no significant difference within the first 24 hours of growth between McIlvaine's solution of pH 3.2 and pH 3.6 (see Table 1). As the cells recovered more or less their normal state, the lower pH was not so favorable and the pH 3.6 showed the most favorable results. As has been mentioned in a previous paper (2), the period from 48 hours to 72 hours shows a tendency to level off. This may be due to deficiency of food supply, growth-promoting substance, and other changes that take place in the artificial environment.

Other factors than the pH factor may be important. It is well known that both distilled water and sucrose produce osmotic effects which in turn affect cell growth. A buffer solution such as McIlvaine's seems a favorable basic medium because of its simplicity, its constant pH, and its other stable characteristics.

From the results discussed, it is evident that if one is to make accurate determinations on the effect of growth-promoting cell-elongation substances it is necessary to control the hydrogen-ion factor as much as possible and, in addition, to control as many other factors as possible, such as the medium, temperature, and humidity. The control of the pH can be achieved by use of a buffered solution similar to that found in the intact plant with a pH as near as possible to that of the normal tissues of the plant. The results obtained by using such a basic medium for testing other substances would insure that

the applied cell-elongation substance was the active principle in the resulting growth.

E. Summary

1. It has been found that pH effects are important to the determination of the efficacy of cell-elongation substances on the growth of *Helianthus annuus* seedlings.

2. Different media have different though not very divergent optimum hydrogen-ion concentrations for maximum growth in the region of cell elongation of *Helianthus annuus* seedlings. This may be due to various factors.

3. A buffer medium with a pH as near as possible to that of the tissue of the plant being tested is important in determining the effect of cell elongation substances on the growth by cell enlargement in *Helianthus annuus* hypocotyls.

4. McIlvaine's buffer solution of a pH 3.6 has been found to be a favorable basic medium for use in the modified *Helianthus* test.

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Growth, 1945, **9**, 217-227.

A MODIFIED HELIANTHUS TEST: III. THE EFFECT OF DIFFERENT CONCENTRATIONS OF INDOLE-3-ACETIC ACID ON CELL ELONGATION IN THE HYPOCOTYL OF *HELIANTHUS ANNUUS* SEEDLINGS

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A. INTRODUCTION

It has been established that growth producing factors play a very important part in the different physiological processes in the plant (5). That normal growth in the length of the plant is governed by auxin has been conclusively proven by such workers as Paal (15), Nielsen (13), Beyer (2), Cholodny (4), and Söding (22). Two requisites, however, for this proof were necessary; first, that the auxin be formed above the region where growth in length takes place, and secondly, that there be a quantitative relationship between the growth-promoter and the growth obtained. Van Overbeek (14) for *Raphanus*, Dijkman (6) for *Lupinus*, and Söding (20, 21) for the *Avena* have shown that a direct proportionality does exist between the growth-substance applied and the resulting growth. The exact mechanism is not fully understood, but various workers attribute the resulting growth to acidity effects which cause a change in the colloidal state of the cell membrane (17, 24). Frey-Wyssling (10), Gessner (11), Söding (23), and Bonner and Thimann (3) are among those who attribute an indirect influence of growth-promoting material on the resulting elongation, due principally to its action on cell wall plasticity and extension. Whatever the effect is upon the protoplasm, it is well known for such growth as cell enlargement, that the property of rapid and polar transport of the growth substance is important (25). It has been found that hetero-auxin (indole-3-acetic acid) has this property, and for this reason it has been used by many workers.

That *Helianthus annuus* seedlings lend themselves favorably to a study of growth by cell enlargement has been well established by Beck, Schocken, and Donnelly (1). That the auxin is formed above the region where growth occurs has been found to be true by Fliry (9) who revealed that auxin is formed in the plumule, but not in the cotyledons of *Helianthus annuus*. The absence of auxin production in the cotyledons of *Helianthus annuus* seedlings was also found to be true by Beyer (2). There is no new development of auxin upon decapitation and thus the auxin and most of the food factor are provided from the plumule and the cotyledons (9). Auxin is one of the many factors necessary for the ordinary growth process and in its absence normal growth does not occur. Thus in the present work only auxin is to be considered with an understanding that in the hypocotyls of dicotyledons growth is largely apical. Very favorable results have been obtained when known concentrations of auxin have been placed in controlled buffer solution and applied according to the modified *Helianthus* technique (8).

The rôle of concentration of growth-promoting factors in growth by cell enlargement is an important one. The response of stems to a range of concentrations of auxins is actually an optimum curve (26), the optimum concentrations varying for various organs and tissues of the same plant as well as for different plants. Only within the physiological range, that is the range of concentrations normally present in the plant, does an increase in auxin concentration bring about an increase in growth. It is the purpose of this paper to present data showing the results of various concentrations of indole-3-acetic acid on cell elongation in *Helianthus annuus* seedlings employing the modified *Helianthus* test (7). Recognizing the fact that auxin is only one of the factors needed for growth, it is shown in this paper that there is an optimal concentration of auxin for growth by cell elongation in the hypocotyl of *Helianthus annuus*.

B. Methods

Seedlings of *Helianthus annuus*, 90 hours old, 4.5 cm. high, and cultured in a dark-chambered incubator, with a relative humidity of 95 per cent, and a temperature of 27° C. were used in these experiments. The technique used in conducting them was as outlined in a previous paper (7). From previous work (8), it had been found that

TABLE 1

THE EFFECT OF DIFFERENT CONCENTRATIONS OF INDOLE-3-ACETIC ACID ON CELL ELONGATION IN THE HYPOCOTYL OF *Helianthus annuus* SEEDLINGS

*Test solution	No. of plants	Average original length of zone	Average growth in mm.			Average per cent growth			Critical Ratio		
			24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
Control	15	9.73 (9.31-10.42)	11.80 (10.42-13.81)	14.81 (13.23-17.18)	16.45 (14.37-24.27)	21.75 (9.70-44.15)	52.30 (35.22-74.77)	68.11 (41.57-132.91)	Reference solution		
0.1 mg.	16	9.80 (9.08-10.64)	13.02 (11.17-17.86)	15.35 (13.00-19.78)	16.19 (13.44-21.07)	32.25 (15.79-67.85)	57.29 (34.76-85.62)	65.78 (39.16- 98.02)	3.45	1.38	0.43
0.2 mg.	12	9.81 (9.47-10.08)	14.67 (12.25-17.58)	16.69 (13.88-18.29)	18.12 (14.91-20.95)	49.15 (29.21-81.23)	70.11 (44.34-88.35)	84.66 (47.92-111.46)	8.06	4.98	2.91
0.3 mg.	14	9.95 (9.43-10.42)	13.70 (12.55-15.83)	14.41 (13.21-16.02)	14.46 (13.15-16.20)	37.66 (26.77-53.98)	44.82 (32.24-55.94)	45.27 (31.36- 60.02)	6.82	2.79	4.95
0.5 mg.	12	9.57 (8.81-10.05)	12.39 (10.90-12.99)	13.51 (12.11-14.54)	14.06 (12.79-15.37)	29.48 (10.66-43.85)	41.26 (29.20-55.34)	46.90 (29.71- 64.20)	3.26	4.65	4.45
0.8 mg.	21	9.42 (8.85-10.83)	13.09 (11.65-15.36)	14.23 (12.47-17.29)	14.90 (12.33-19.44)	32.47 (14.65-49.00)	43.22 (28.17-59.65)	47.62 (28.47- 79.50)	4.76	3.34	4.17
1.0 mg.	22	10.05 (9.54-10.82)	13.39 (11.81-14.99)	14.84 (13.15-16.62)	15.36 (13.31-17.21)	33.29 (22.51-45.08)	47.79 (32.16-64.33)	53.02 (33.36- 73.13)	5.92	1.66	3.22

*McIlvaine's buffer solution, pH 3.6 used as control.
McIlvaine's buffer solution, pH 3.6 used with various concentrations of indole-3-acetic acid.

TABLE 2

A COMPARISON OF THE EFFECT OF 0.1 MG. AND 0.2 MG. OF HETEROAUXIN PER LITER OF McILVAINE'S BUFFER SOLUTION, pH 3.6, ON THE ZONE OF CELL ENLARGEMENT IN THE HYPOCOTYL OF *Helianthus annuus* SEEDLINGS

Concentration in liter of McIlvaine's	No. of plants	Average original length of zone	Average increase in length in mm.			Average per cent growth			Critical D/E_D Ratio		
			24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
0.1 mg	16	9.80 (9.08-10.64)	13.02 (11.17-17.86)	15.35 (13.00-19.78)	16.19 (13.44-21.07)	32.25 (15.79-67.85)	57.29 (34.76-85.62)	65.78 (39.16- 98.02)	4.40	3.29	3.99
0.2 mg.	12	9.81 (9.47-10.08)	14.67 (12.25-17.58)	16.69 (13.88-18.29)	18.12 (14.91-20.95)	49.15 (29.21-81.23)	70.11 (44.34-88.35)	84.66 (47.92-111.46)			

McIlvaine's buffer solution, pH 3.6, was a favorable basic medium for use in the modified *Helianthus* test. For this reason, the varying concentrations of indole-3-acetic acid (Eastman Kodak Co.) used in these experiments were made up in this buffer solution. The control group was run with plain McIlvaine's buffer solution, pH 3.6, and the dilutions of indole-3-acetic acid tested were 0.1, 0.2, 0.3, 0.5, 0.8, and 1.0 mg. per liter of McIlvaine's solution at pH 3.6. After the initial preparation of the seedlings the zone of cell enlargement was marked in each plant from the cut surface of the seedlings to the index needle (*Bivonea stimulosa,* inserted epidermally approximately 10 mm. below the cut surface). By means of the horizontal measuring microscope, readings were taken of this zone every 24 hours for three consecutive days.

C. Experimental

The results obtained with the varying dilutions of indole-3-acetic acid in McIlvaine's buffer solution, are recorded in Table 1. The average length of the original zone of cell enlargement used in each series of test and control plants; the average increase in length of this zone; the average per cent increase in length; and the critical ratios for each test group as compared with the control were determined. Any plant manifesting bending, mechanical injury, or variation from the mean by more than 10 per cent was discarded. The critical ratios were determined to find the significant points of difference in this series of experiments. The critical ratio between the most favorable concentration of indole-3-acetic acid and the next most favorable dilution was likewise determined for each period of measurement to prove the significance of the results as shown in Table 2. The critical ratio was determined in each case by means of the formula:

$$D/E_D = \frac{\textit{Difference of the Means } (D)}{0.6745\sqrt{\frac{\sigma_1^2}{n_1 - 1} + \frac{\sigma_2^2}{n_2 - 1}}}$$

An analysis of these results can be seen more clearly from Figures 1 and 2 which give a more vivid representation of the results obtained. In Figure 1 the percentage increase in growth is plotted against the concentrations of indole-3-acetic acid, and in Figure 2, the percentage

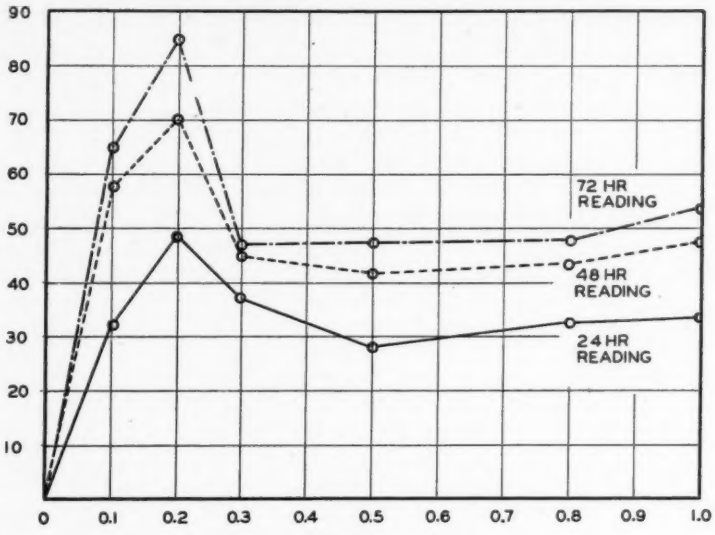

FIGURE 1

COMPARISON OF DIFFERENT CONCENTRATIONS OF INDOLE-3-ACETIC ACID IN MG. PER LITER OF McILVAINE'S BUFFER (pH 3.6)

(The abscissa is concentration of indole-3-acetic acid in McIlvaine's buffer, the ordinate is percentage increase in growth over original length.)

increase in growth is plotted against time in hours. The best concentration is seen at the peak of Figure 1 which represents an optimum concentration of 0.2 mg. of indole-3-acetic acid per liter of McIlvaine's buffer solution. According to Figure 2, a concentration of 0.1 mg. per liter of McIlvaine's is the next favorable concentration of indole-3-acetic acid. That 0.2 mg. is significantly the best, however, is shown by the critical ratios between these two concentrations presented in Table 2. A concentration of 1 mg. per liter according to Table 1 does not produce significant results at the 48-hour period but at the 24-hour reading it causes greater initial growth than the control. An additional day's growth brings the 1 mg. per liter concentration near the control (within experimental error), and on the third day it falls below the control as shown in Figure 2, thus revealing a depressing effect. In contrast to this depressing effect of

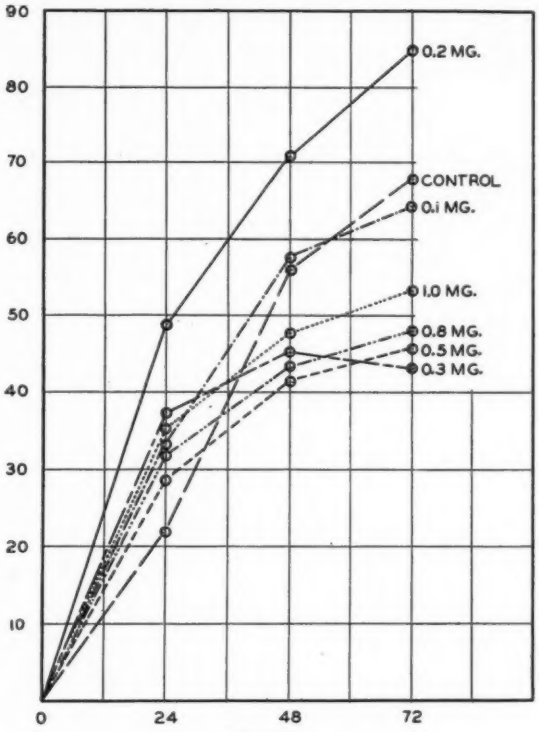

FIGURE 2

COMPARISON OF DIFFERENT CONCENTRATIONS OF INDOLE-3-ACETIC ACID IN MG. PER LITER OF MCILVAINE'S BUFFER (pH 3.6)

(The abscissa is in hours, the ordinate is in percentage of increase in growth over original length.)

all the higher concentrations (namely, the 1.0 mg., the 0.3 mg., the 0.5 mg., and 0.8 mg. per liter of McIlvaine's buffer solution), the 0.1 mg. dilution of indole-3-acetic acid is above the control at 24 hours but at the 48- and 72-hour periods, it is equal in effect to the control, showing no significant difference. Thus we see that an initial impetus to growth is given but thereafter the effect parallels the control within experimental error.

D. Discussion and Conclusion

It seems proper to emphasize the advantage of using *Helianthus annuus* seedlings in testing the effect of auxins on cell elongation. As Dijkman (6), Strugger (24) and others have pointed out, there is no regeneration of the center of auxin production in decapitated *Helianthus* seedlings, and therefore, no chance for naturally occurring growth-promoting substances to interfere with the substance being tested. Ninety-hour-old seedlings (4.5 cm. high) were used, since the age of the plant is an important factor in so far as response to indole-3-acetic acid (27, 1) is concerned.

From the data presented it has been demonstrated that the concentration of indole-3-acetic acid and duration of application are important in affecting cell-elongation in *Helianthus annuus* seedlings. It has been shown that an optimum concentration exists for the growth response. The initial effect of the growth-promoter upon the zone of cell enlargement during the first 24 hours is significant in most of the tested concentrations due to the presence of the substance itself; that is, the effect on growth is not a concentration effect except in the case of the optimal concentration of 0.2 mg. per liter of solution as seen in Figure 2. The other concentrations are not significantly different (Figure 2 and Table 1). In the case of 0.1 mg. of indole-3-acetic acid per liter of solution the initial impetus of growth is likewise manifested but thereafter the effect parallels the control within experimental error. This is no doubt due to lack of sufficient growth-promoting factor because the external appearance of the plants tested with this low concentration manifested a straight but slow normal growth appearance, whereas the plants tested with the higher concentrations of the heteroauxin had a tendency to show nastic movement and presented an abnormal appearance. This difference in response may be due to a number of various factors. For example, it is well known that the physiological condition of the cell determines the action of the growth factor (16, 12) and that different tissues of the same plant have different auxin-responses (26) as well as different rates of supply of nutrients, water, and growth-promoting substances.

The optimum concentration of 0.2 mg. of indole-3-acetic acid per liter of McIlvaine's buffer solution obtained by using the modified

Helianthus test is in agreement with the optimal concentration for this compound in the *Avena* test (28).

It is worthy to note, however, that preliminary experiments in these laboratories showed that in Knop's solution, the optimal concentration of heteroauxin is 0.5 mg. This indicates that McIlvaine's buffer solution is a better medium as far as the sensitivity to the indole-3-acetic acid is concerned in the modified *Helianthus* test. For this reason, we are using McIlvaine's buffer solution (pH 3.6) as the basic medium in testing various plant and yeast extracts with the modified *Helianthus* test.

As has been stated in the previous work of Fardon, Maynard, and McDowell (7), the 48-hour period in the modified *Helianthus* test seems to be a favorable period for noting the growth-promoting effect of substances. Comparison of the critical ratio of the 24-hour period (4.98) and the 72-hour period (2.91) from data obtained with the optimal concentration of 0.2 mg. indole-3-acetic acid confirms this opinion. The 72-hour period is of interest, however, because the higher concentrations depress the growth significantly below that of the control. It is, therefore, advisable to conduct such experiments over a period of 72 hours in certain cases in order to determine the depressing or stimulating effect which does not become evident in some cases until this period is reached. As can be seen in Figure 2, the general trend of the tested substances can be determined at the 48-hour period because the effect either tends to be above or below that of the control at that period. The toxicity due to high concentrations of growth-promoting substance as seen in these experiments is in accordance with that found by other workers (18, 28).

A quantitative relationship between the amount of heteroauxin applied and the resulting growth by cell enlargement in the hypocotyl of *Helianthus annuus* seedlings is seen to exist within proper limits of the concentration of the growth-promoter, provided optimum growing conditions prevail throughout the period of experimentation. Under carefully controlled experimental conditions two independent factors are found to limit cell enlargement in the hypocotyl of *Helianthus annuus* seedlings; either an insufficient amount of the growth-promoter or an excess of it. In the first case, a retardation of growth is observed, while in the second case an abnormal type of growth characteristic of toxicity effects is obtained. It is of importance to note

also that in the plant under discussion, the activation of growth is brought about by low concentrations of heteroauxin comparable with those concentrations found in the normal plant (19).

E. Summary

1. The zone of cell enlargement, beginning 5 mm. below the base of the cotyledons and extending approximately 10 mm. in length, in 90-hour-old *Helianthus annuus* seedlings (45 mm. high) responds favorably to varying concentrations of indole-3-acetic acid within certain limits.

2. The most favorable concentration of indole-3-acetic acid for growth in this zone is 0.2 mg. per liter of McIlvaine's buffer solution adjusted to a pH of 3.6.

3. An inhibitory effect is observed in higher concentrations of indole-3-acetic acid (0.3; 0.5; 0.8; and 1.0 mg. per liter of McIlvaine's buffer solution, pH 3.6) as shown by the external appearances of the seedlings as well as by actual measurement.

4. A concentration of 0.1 mg. per liter of indole-3-acetic acid in McIlvaine's buffer solution reveals initial growth activity but approximates the control growth with McIlvaine's buffer solution, pH 3.6, after the 24-hour period more closely.

5. The 48-hour period in the modified *Helianthus* test is a sufficient length of time if an indication of the growth effect is desired; the 24-hour period is an insufficient length of time due to the initial impetus indicated in every case; the 72-hour period should be chosen if a definite stimulation or depression of growth is to be verified.

6. The modified *Helianthus* test has been found reliable and accurate in measuring the effects of other extracts and substances by comparing the growth in cell enlargement to the various concentrations of indole-3-acetic acid.

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